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# Membrane Transport and Cytotoxicity of Nucleoside Analogs in Acute Leukemia Cells

by

Shivani Bhardwaj

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pharmacology

Edmonton, Alberta

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31 JULY 2003

# ABSTRACT

Clinical resistance to antileukemic nucleoside drugs is poorly understood. Most nucleosides depend on membrane transport processes for entry into cells. This study investigated whether *es* nucleoside transporter-mediated membrane fluxes of nucleosides are determinants of their cytotoxicity in leukemic blasts from patients. Cytotoxicity and inward fluxes of cytarabine and gemcitabine were measured in freshly-isolated blasts from patients with leukemia, and in human leukemic myeloblast KG1 cells. Nucleoside influx and cytotoxicity were reduced in the presence of nitrobenzylthioinosine, an *es* nucleoside transport inhibitor. *Es* transporter-mediated nucleoside influx and cytotoxicity varied among patient samples, and were correlated for cytarabine ($r=0.51$, $p<0.05$) but not for gemcitabine. Furthermore, selection and characterization of an *es* nucleoside transporter-deficient, gemcitabine-resistant leukemic T-lymphoblast cell subline demonstrated that nucleoside drug resistance was primarily attributable to *es* transporter-deficiency. These results suggest that *es* nucleoside transporter activity is a determinant of nucleoside cytotoxicity and that transport deficiency may be a mechanism of resistance to nucleoside therapy in leukemia.

*Dedicated to my parents for their love and support*

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## List of Abbreviations

| | |
|---|---|
| 2-CdA | 2-chlorodeoxyadenosine |
| araC | arabinosylcytosine |
| araU | arabinosyluracil |
| araCMP | arabinosylcytosine monophosphate |
| araUMP | arabinosyluracil monophosphate |
| ATRA | all-trans retinoic acid |
| BMT | bone marrow transplantation |
| *cib* | concentrative nucleoside transport process, insensitive to NBMPR, broad substrate specificity |
| *cif* | concentrative nucleoside transport process, insensitive to NBMPR, transports formycin B |
| *cit* | concentrative nucleoside transport process, insensitive to NBMPR, transports thymidine |
| CLL | chronic lymphocytic leukemia |
| CML | chronic myeloid leukemia |
| cpm | counts per minute |
| CR | complete remission |
| CRT | cranial irradiation |
| CSF | cerebrospinal fluid |
| dCK | deoxycytidine kinase |
| dCTP | deoxycytidine 5′-triphosphate |

| | |
|---|---|
| dFdC | 2′,2′-difluorodeoxycytidine |
| dFdCMP | 2′,2′- difluorodeoxycytidine monophosphate |
| dFdCMP | 2′,2′-difluorodeoxycytidine diphosphate |
| dFdCMP | 2′,2′-difluorodeoxycytidine triphosphate |
| dFdU | 2′,2′-difluorodeoxyuridine |
| DMSO | dimethylsulphoxide |
| DPM | dipyridamole |
| DNA | deoxyribonucleic acid |
| DThd | thymidine |
| DTT | dithiothreitol |
| *ei* | equilibrative nucleoside transport process, insensitive to NBMPR |
| *es* | equilibrative nucleoside transport process, sensitive to NBMPR |
| FAB | French American British system for classification of acute myeloid leukemia and myelodysplastic syndromes |
| FACS | fluorescence activated cell sorting |
| FBS | fetal bovine serum |
| G-CSF | granulocyte colony-stimulating factor |
| HD-MTX | high dose methotrexate |
| HEPES | N-[2-Hydroxyethyl]piperazine-N′-[2-ethane sulfonic acid] |
| hr | hour |
| HSC | buffer containing 10 mmol/l HEPES buffer, 140 mmol/l of NaCl, 2.5 mmol/l of $CaCl_2$, pH 7.4 |
| $IC_{50}$ | 50% inhibitory concentration |

| | |
|---|---|
| MTX | methotrexate |
| NaF | sodium fluoride |
| NBMPR | nitrobenzylthioinosine; 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosyl-purine |
| PBSA | phosphate buffered saline solution A; 137 mmol/l NaCl, 8 mmol/l $Na_2HPO_4$, 1.5 mmol/l $KH_2PO_4$, 2.7 mmol/l KCl, pH 7.4 |
| PI | propidium iodide |
| pmol | picomole (s) |
| RFI | relative fluorescence intensity |
| R/H/10F | RPMI 1640 medium containing 2 mmol/l HEPES buffer and 10% FBS, pH 7.4 |
| RPMI | Roswell Park Memorial Institute |
| $RPMI^t$ | RPMI 1640 medium for transport experiments (RPMI medium with 100 mmol/l HEPES buffer and 48 mmol/l NaCl, pH 7.4) |
| SAENTA | 5′-*S*-(2-aminoethyl)-$N^6$-(4-nitrobenzyl)-5′-thioadenosine |
| SD | standard deviation |
| Sec | second |

# Chapter 1

# INTRODUCTION

Nucleoside analogs such as cytarabine (arabinosylcytosine, araC), 2-chlorodeoxyadenosine (2-CdA), fludarabine and 5-azacytidine are important agents in the treatment of leukemia. In spite of the use of a number of drugs not all patients respond to therapy, and many who respond initially will later relapse with drug resistant disease. A number of potential mechanisms of drug resistance have been studied in experimental systems but the basis of clinical resistance to antileukemic nucleoside drugs is not well understood. This thesis is concerned with one possible mechanism of resistance to nucleoside drugs in leukemic blasts from patients; that is, a deficiency in membrane transport of nucleosides.

**1.1 Leukemia and its treatment**

The term leukemia refers to a group of disorders in which proliferation and maturation of leucocytes is no longer under the normal physiological mechanisms of control, leading to an abnormal proliferation of white blood cells.

Acute myelogenous leukemia (AML) is a result of neoplastic transformation of myeloid precursors with a marked reduction in the capacity for differentiation into mature cellular elements. AML occurs predominantly in adults and its incidence increases with age. Current chemotherapy strategies result in complete remission rates of 50-80%. Improvements in the treatment of AML may be attributed to the use of more effective drug combinations and accurate diagnosis. AML is classified into different subtypes according to the FAB classification scheme. Subtype M0 is characterized by absence of morphologic myeloid proliferation.[1] In subtype M1 (acute myeloblastic leukemia with minimal maturation) the predominant cells, which represent 90 percent of the nonerythroid cells are the poorly differentiated myeloblasts. Subtype M2 is characterized

by significant maturation in all cells beyond promyelocyte stage. AML subtype M3 is a distinct class of AML that is characterized by the proliferation of promyelocytes. M4 is a mixture of granulocytic and monocytic lineage and M5 is monocytic lineage. M6 is characterized by granulocytic proliferation associated with dysplastic erythroid proliferation. Subtype M7, acute megakaryoblastic leukemia is a rare form of AML associated with blasts or clusters of megakaryoblasts and a decrease in myeloid maturation.

The treatment of AML is designed with the intention of producing durable remission with rapid restoration of normal bone marrow functions. Treatment of AML is traditionally divided into two parts: a) treatment given to restore the bone marrow to normal, termed remission induction therapy and b) treatment given once the remission has been achieved and referred to as consolidation or post remission therapy. It is generally assumed that a few leukemia cells persist after the remission induction therapy (minimal residual disease), which causes the relapse of the disease. Consolidation therapy consists of one or more courses of chemotherapy or bone marrow transplantation to eliminate the minimal residual disease, thus producing a durable remission. Cytogenetic studies are performed before the initiation of the therapy, which have prognostic significance and guide the therapy.

The aim of induction therapy is to eradicate the malignant clone of leukemic cells and also to restore normal hematopoiesis. AraC has been a mainstay in induction therapy of AML. Most remission induction systems employ araC in combination with an anthracycline. The combination of an anthracycline such as daunorubicin or idarubicin for three days, with araC, seven days (also called "7+3" regimen) is the most common

remission induction regimen employed today.[2] Increasing the dose of cytarabine or the addition of other chemotherapeutic drugs such as etoposide to improve the response rates have also been evaluated.[3] Other drugs, which have been used in combination with araC, include mitoxantrone, an anthraquinone derivative. Hematopoietic growth factors are also often employed in treatment of AML. These growth factors are used to ameliorate chemotherapy-induced myelosuppression and potentially put the leukemic cells into cycle before the administration of araC.

Post remission treatment includes consolidation chemotherapy or bone marrow transplantation (allogeneic or autologous). The consolidation chemotherapy includes the drugs employed in remission induction repeated for one or more cycles. Allogeneic bone marrow transplantation provides durable long-term disease free survival in patients in first complete remission. Autologous bone marrow transplantation is employed when patients lack an allogeneic bone marrow donor but its role remains controversial.

Myelodysplastic syndrome (MDS) is a heterogeneous group of hematological disorders characterized by peripheral blood cytopenias with a hypercellular bone marrow exhibiting dyshematopoiesis. It occurs predominantly in adults, often after chemotherapy and may progress to AML.[4] The clinical course of MDS is quite variable. The goals of MDS therapy include a) reducing the risk of leukemic transformation, b) control of symptoms due to cytopenias, c) improving overall survival and d) reducing the toxicity of therapy and ultimately improving quality of life. Cytokines, G-CSF (granulocyte colony-stimulating factor) and erythropoietin have been used to ameliorate the cytopenias.[5] Differentiating agent, all-trans retinoic acid (ATRA) has also been employed in the treatment of MDS with low response rate of 10%.[6] Chemotherapy for MDS, once

transformed includes araC and idarubicin protocols, which have resulted in response rates between 48%-58%[7, 8] but these are typically transient.

Currently allogeneic bone marrow transplantation may be offered to MDS patients with poor short-term prognosis, in particular patients who are younger than 55 years of age. The disease free survival at 3 years is approximately 40%; treatment related mortality lies between 25-45% and the relapse rate is 8-34%.[9]

Acute lymphoblastic leukemia (ALL) is the clonal proliferation of transformed lymphoid progenitor cells in the bone marrow and is the most frequent neoplastic disease of childhood. ALL accounts for 20 percent of acute leukemia in adults[10] and 80 percent of acute leukemia of childhood.[10, 11] Substantial advances in the treatment of ALL have been made in the past 10-15 years. With the current chemotherapy and supportive care, 70 to 80 percent of children achieve a durable complete remission (CR), whereas the response rates in adults are lower.

The use of multiple chemotherapeutic agents has greatly improved the prognosis of patients with ALL. Current therapy of ALL is divided into four phases: remission induction, intensification/consolidation, CNS prophylaxis and maintenance therapy. The majority of standard induction regimens for ALL include prednisone, vincristine and L-asparginase.[12] Other drugs such as anthracyclines and araC also supplement the standard induction regimen drugs.[13] Colony stimulating factors such as G-CSF are often employed in the treatment of ALL for shortening the period of neutropenia post induction to reduce infection risk. Identification of the different risk groups in pediatric ALL has led to the intensification of chemotherapy for intermediate and high-risk groups.

After the remission induction multiple courses of potentially non-cross resistant drugs such as araC, MTX, L-asparginase, doxorubicin and cyclophosphamide are employed in the consolidation regimens. Hematopoeitic cell transplantation is also used for sibling patient in the consolidation phase of treatment. CNS prophylaxis is an essential part of ALL treatment as it leads to a reduced rate of CNS and bone marrow relapse. CNS prophylaxis can be achieved in a number of ways such as a) intrathecal administration of MTX or araC, b) cranial irradiation (CRT) and c) systemic high dose MTX or high dose araC, so that cytotoxic levels in the CSF are achieved.[14] ALL maintenance protocols employ MTX with monthly doses of prednisone or vincristine. The optimal duration of the maintenance treatment is however unknown.

**1.2 Resistance associated with leukemia**

Despite of the availability of numerous drugs for the treatment of leukemia, resistance is still a major challenge. Drug resistance of leukemic cells leads to refractory leukemia or recurrence. For patients with AML, who fail to respond to the induction therapy or relapse, current therapy is insufficient, with disappointing response rates, often less than 10%. Several mechanisms of drug resistance have been described in patients with leukemia but none have been established as a basis of clinical resistance. In a study performed by Wiley *et al.*, a six-fold range in arabinosylcytosine triphosphate (araCTP) accumulation in various leukemic blasts from patients was studied, and it was observed that at low concentrations of araC, membrane transport was the rate-limiting step in the formation of araCTP in leukemic blasts from patients.[15] A strong correlation between the maximum density of [$^3$H]NBMPR binding sites and araCTP accumulation was observed. Other studies implicating membrane transport in the cytotoxicity of nucleoside analogs

such as araC[16, 17] (in leukemic blasts from patients) and dFdC[18] (in leukemic cell lines) have been performed. A deficiency of the *es* nucleoside transporter as a potential mechanism of araC resistance in patients[16] has also been suggested. Other mechanisms of resistance to araC that involve expression of efflux pumps or cytidine deaminase have also been studied.[19] However, none of these mechanisms have been established as basis of clinical resistance.

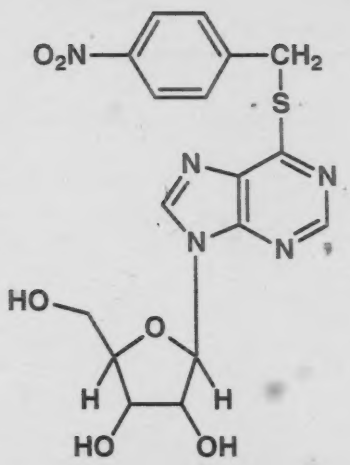
**1.3 Nucleoside transporters**

The pharmacologic target of anticancer nucleoside analogs is intracellular; therefore their transport across the cell membrane is a requisite for their cytotoxic activity. The antileukemic nucleoside analogs are hydrophilic molecules.[16] The entry of these agents into cells is mediated by nucleoside specific transporters that facilitate their transmembrane fluxes.[20, 21] Nucleoside specific membrane transporters have been described in many cell types. These nucleoside transporters are determinants of the accumulation and retention of anticancer nucleosides in leukemia cells.[17] In studies of nucleoside transporters, most of the focus has been on inwardly directed transport processes but these nucleosides are also released from the cells through processes that function in the opposite direction. Some of the antiviral nucleoside drugs such as didanosine, zidovudine and lamivudine have been shown to be the substrates of nucleoside transporters.[22] The presence or absence of these nucleoside transporters will have an important impact on the pharmacokinetics, distribution and the *in vivo* biological activity of nucleoside analogs.[23] Nucleoside transporters are promising targets in cancer chemotherapy as by the manipulation of the nucleoside transporter activity, nucleoside

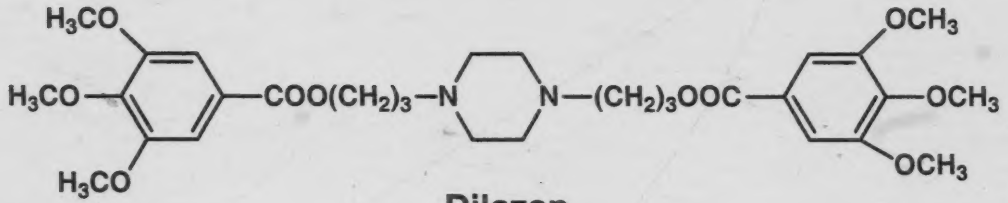
anticancer drug resistance could be studied. It has been suggested that these nucleoside transporters may play a role in resistance to nucleoside drugs.[24]

Nucleoside transporters are classified based upon the functional and pharmacological characteristics of membrane fluxes.[21] To date; seven different nucleoside transporter subtypes have been documented. These nucleoside transporters have been categorized into two distinct groups based upon the transport mechanisms: a) the equilibrative nucleoside transporters, which are driven solely by concentration gradient of the nucleoside permeants and b) the concentrative nucleoside transporters, which are driven by the transmembrane sodium gradients.[25] The equilibrative nucleoside transporters have broad permeant selectivities and are found in most cell types. Also, the equilibrative nucleoside transporters mediate both the influx and efflux of nucleosides. However, no data are available which show the concentrative nucleoside transporters mediating nucleoside fluxes in the outward direction. The concentrative nucleoside transporters are found in a few epithelial cell types and have narrow permeant selectivity as compared to the equilibrative nucleoside transporters.[22] However, two or more nucleoside transporters may be co expressed in some cell types.[20]

***1.3.1 Equilibrative nucleoside transporters.*** The equilibrative nucleoside transporters are further classified on the basis of sensitivity to nitrobenzylmercaptopurine ribonucleoside (NBMPR, nitrobenzylthioinosine). NBMPR (Figure 1) is a tightly binding and selective inhibitor of nucleoside transport.[23] NBMPR has been extensively employed as a tool for characterization of nucleoside transport processes in mammalian cells.[21, 23] The equilibrative nucleoside transporter, which is sensitive to nanomolar




**Nitrobenzylmercaptopurine ribonucleoside (Nitrobenzylthioinosine)**

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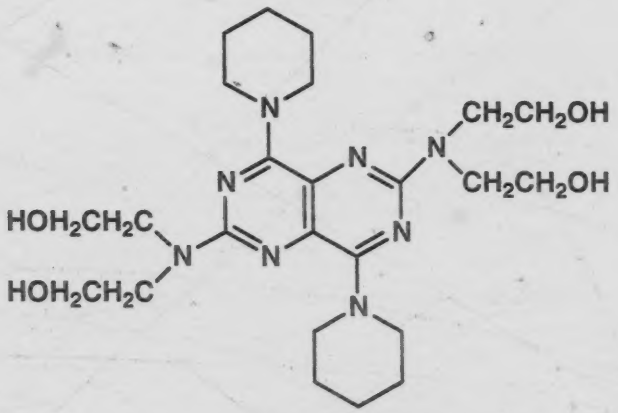



**Dipyridamole**

**Figure 1: Structures of nucleoside transport inhibitors studied in this project.**

concentrations of NBMPR, is designated as *es* (equilibrative, NBMPR sensitive) nucleoside transporter, whereas the other subtype is insensitive to nanomolar concentrations of NBMPR but inhibited at micromolar concentrations of NBMPR[20] and is designated as *ei* (equilibrative, NBMPR insensitive). NBMPR binds reversibly at sites on the *es* nucleoside transporter[28] and has a high affinity ($K_d$ = 0.1-1 nM). The NBMPR binding site has been shown to be located at the extracellular face of the plasma membrane and corresponds to the nucleoside permeation site.[27] The *es* nucleoside transporter was first characterized in erythrocytes by Oliver and Paterson, who demonstrated its sensitivity to NBMPR and related compounds.[28] A number of studies have been conducted which employed [$^3$H]NBMPR in radioligand binding assays to measure the content of *es* nucleoside transporters in leukemia cells from patients and cultured cells.[15, 17, 29] AraC influx was found to be correlated with the number of [$^3$H]NBMPR binding sites.[29]

The other subtype of equilibrative nucleoside transporter, the *ei* nucleoside transporter, was recognized by its relative insensitivity to NBMPR, which inhibits the *ei*-mediated fluxes in the micromolar concentration range.[20] *Ei* is typically found in cells and tissues together with other nucleoside transport processes.[22]

***1.3.1.1 Molecular properties of equilibrative nucleoside transporters***. Equilibrative nucleoside transport processes are widely distributed in mammalian tissues. The cloning of first human equilibrative nucleoside transporter (hENT1) from human placental library was in 1997.[30] After the expression of hENT1 in *Xenopus* oocytes, its kinetic characterization showed it to be an *es*-type transporter with broad substrate selectivity for purine and pyrimidine nucleosides. It has been shown to transport nucleoside analogs

such as araC, 2-CdA and dFdC and is inhibited by NBMPR, dipyridamole (DPM) and dilazep.[30] The hENT1 nucleoside transporter has a sequence of 456 amino acids (50 kDa) and has been shown to have 11 transmembrane domains along with three potential N-linked glycosylation sites.

After the identification of hENT1, the cloning of cDNA encoding a human *ei* type transporter was performed quickly. The human cDNA encoding hENT2 protein (36 kDa, 456 amino acids) are ~50% identical to their ENT1 counterparts.[31] Like hENT1, hENT2 possesses three potential glycosylation sites on the extracellular face of the protein, [32] of which two are found in the predicted extracellular loop between transmembrane domains 1 and 2 and one is found in the predicted intracellular loop between transmembrane domains 6 and 7. When expressed in *Xenopus* oocytes, hENT2 was found to exhibit the properties of mammalian *ei* type transport processes, which were less sensitive to inhibition by NBMPR than hENT1.[33] hENT2 transports a broad range of purine and pyrimidine substrates and unlike hENT1, hENT2 transports zidovudine and nucleobases such as hypoxanthine.[33] Recently, cDNA sequences encoding a third mammalian ENT family member from human (hENT3) and mouse (mENT3) cells have been reported.[31] The hENT3 transporter (475 amino acids) has been shown to be 73% identical in sequence to the mouse, rat and human ENT1 and ENT2 transporter isoforms. When the transcripts encoding the putative hENT3 or mENT3 protein were injected into oocytes, nucleoside uptake was not detected.[31] The substrate selectivity of m/hENT3 has not yet been established.

***1.3.2 Nucleoside transport inhibitors***. Apart from NBMPR, other non-nucleoside compounds such as dilazep and DPM (Figure 1), which were initially recognized as

vasodilatory and antiplatelet agents, inhibit both *es* and *ei* nucleoside transporters.[34] However it is known that the *es* nucleoside transporter is more sensitive to DPM and dilazep as compared to the *ei* transporter.[34] Sensitivity of hENT1 to inhibition by dilazep and DPM has been demonstrated with $IC_{50}$ values of 60 ± 2 nM and 140 ± 2 nM respectively.[34] However the results with clinical studies employing DPM have been dissapointing.[35] The failure of DPM to augment the selectivity of antimetabolites has been attributed to the heterogeneity of nucleoside transport in mammalian cells.[20] DPM has been shown to potentiate the cytotoxic activity of araC by preventing its excretion from the cell. An augmentation of araC cytotoxicity by inhibiting drug efflux from human ovarian carcinoma (2008) and promyelocytic leukemia (HL60) cells was observed by employing DPM as transport inhibitor.[36] In this study, ovarian cancer 2008 cells and HL60 cells were treated with 20 μM DPM, 2 hours after exposing the cells to graded concentrations of araC. It was observed that the cytotoxicity of the nucleoside analog increased 100-300% at all drug concentrations tested. However, the use of DPM as a transport inhibitor has some drawbacks. The concentration of DPM used in most of the studies is 5-50 μM. Only 5-10% of the drug is protein bound in tissue culture medium, so that the amount of free DPM is large. In contrast, DPM is 95% protein bound *in vivo* and high concentrations of free drug are hard to achieve clinically.

Dilazep is also one of the most potent non-nucleoside inhibitors of the *es* nucleoside trasnporter. Dilazep hydrochloride has high aqueous solubility and is employed as a transport-stopping agent in the measurement of nucleoside fluxes in cells that possess NBMPR sensitive transporters.[23] Congeners of mioflazine such as draflazine have also been recognized as inhibitors of the *es* nucleoside transporter.[16, 37]

***1.3.3 A flow cytometric probe for the es nucleoside transporter: SAENTA-fluorescein.*** The determination of *es* nucleoside transporter abundance in cells frequently employed [$^3$H]NBMPR in equilibrium binding studies in the past. This method was used for the determination of *es* transporter content in both cultured cells and leukemia cells from patients.[17, 29] This procedure had some drawbacks, as it requires a large number of cells and is time consuming. The synthesis of SAENTA (5′-S-(2-aminoethyl)-$N^6$-(4-nitrobenzyl)-5′-thioadenosine) led to the development of a sensitive and facile method of determining the cellular *es* transporter abundance. SAENTA is a derivatizable analog of NBMPR and is a tightly bound, useful probe of the *es* transporter.[38] SAENTA-fluorescein (Figure 2) is synthesized by reacting a fluorescein derivative with SAENTA to give 5-(SAENTA-x8)-fluorescein.[39] Several conjugates of SAENTA with fluorescein moieties have been developed and used for studies of *es* transporters.[39] This development of SAENTA-fluorescein allows flow cytometric enumeration of *es* transporters and requires fewer cells than the standard [$^3$H]NBMPR assay. When SAENTA-fluorescein is employed in equilibrium binding assays, a $B_{max}$ value, which is a measure of the cellular content of *es* transporters, can be determined by flow cytometry.

***1.3.4 Concentrative nucleoside transporters.*** Concentrative nucleoside transporters are also present in the plasma membranes of certain cells.[21] These concentrative nucleoside transporters have been found only in certain tissue types including intestinal and renal epithelia, liver[23] and in leukemic cells.[40] The concentrative nucleoside transporters mediate the uptake of nucleosides through sodium/nucleoside co-transport system. Here, the inward flux of nucleosides is coupled to inward movement of sodium.[41] The requirement for sodium is specific for these transporters, although one study reported that

# 5-(SAENTA-x8)-fluorescein
# ("SAENTA-fluorescein")

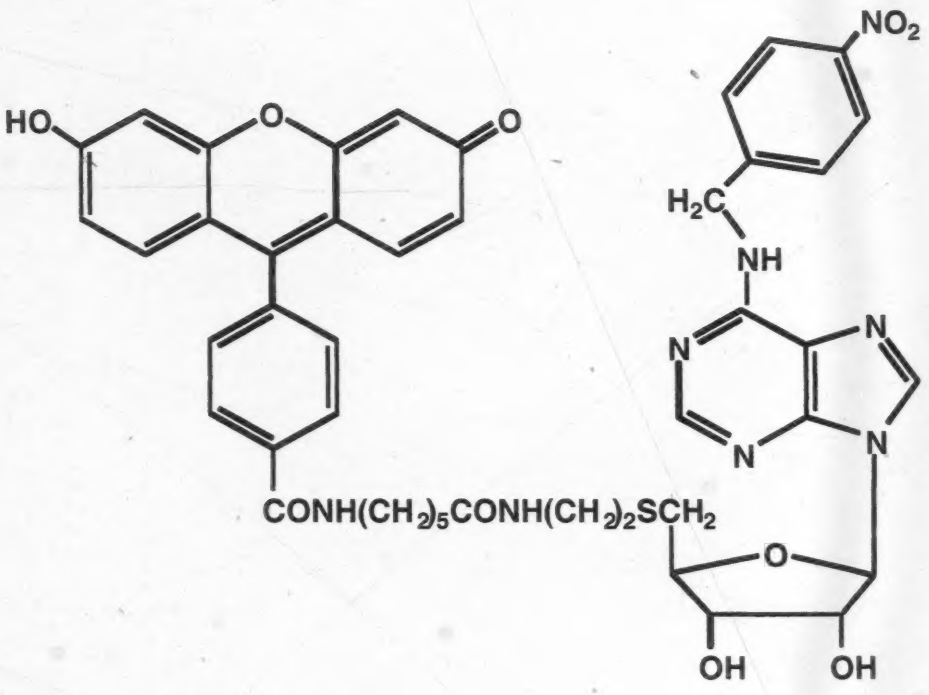



**Figure 2: Structure of SAENTA-fluorescein.**

*Adapted from*: Gati, W.P., Paterson, A.R., Belch, A.R., Chlumecky, V., Larratt, L.M., Mant, M.J., Turner, A.R. *Es* nucleoside transporter content of acute leukemia cells: role in cell sensitivity to cytarabine (araC). *Leukemia & Lymphoma.* 32(1-2): 45-54, 1998

$Li^+$ was as effective as $Na^+$ in rat liver and canalicular membrane vesicles.[42] These nucleoside transporters are typically insensitive to NBMPR. A sodium-linked transport system was identified in cultured mouse leukemia L1210 cells.[43] It was observed that the presence of nucleoside transport inhibitors (DPM), led to an increased accumulation of nucleosides (9-β-D-arabinofuranosyladenine) in the cells compared to the extracellular concentrations.[43] This accumulation led to an increase in the sensitivity of L1210/C2 cells to the nucleoside in the presence of DPM. The accumulation could be accounted for by a) the blockade of equilibrative nucleoside transporters by the transport inhibitor, which mediated efflux of the nucleosides, and b) to the uptake of nucleosides by a concentrative transporter, which was insensitive to the transport inhibitor. However, high concentrations of DPM (> 50 μM) may cause some inhibition of $Na^+$-dependent nucleoside transporters.[44] Some studies have employed transport inhibitors such as lidoflazine and analogs but no inhibition of concentrative nucleoside transport was observed.[45] Dilazep has also been employed in a number to studies to study its effect on concentrative nucleoside transporters.[45]

Two systems of nomenclature for concentrative, $Na^+$-linked nucleoside transporters are in use. The first involves numerical designations (N1-N5), whereas the second distinguishes between the concentrative transporters based on their functional properties, including substrate selectivity. Thus, the transporters are designated as follows: (a) *cit*, a process that accepts adenosine (subtypes N2 and N4) or guanosine (subtype N4) as a permeant, but not formycin B, (b) *cif*, (subtype N1) exhibits preference for purine nucleosides and accepts uridine (subtype N1) and formycin B as permeants, (c) *cib*, has a

broad range of permeant selectivity, accepting both pyrimidine and purine nucleosides (subtype N3), and d) *cs* (N5), a concentrative, $Na^+$ linked process that is sensitive to low concentrations of NBMPR.[20] However, the permeant selectivity of *cs* transporter still has to be determined. Three different transporter proteins have been described that are responsible for *cit* (hCNT1), *cif* (hCNT2), and *cib* (hCNT3) activities. The *cif* transporter is found in normal cells[21] as well as neoplastic cells such as L1210 murine leukemia.[43] The *cit* transporter has been observed in fresh tissue preparations from kidney epithelia and intestine.[21] The *cib* transporter has been identified in rat jejunum, rabbit choroid plexus and in human myeloid leukemic cells and in freshly isolated myeloblasts.[46] The *cs* nucleoside transporter has been identified in freshly isolated leukemic cells from patients with AML, ALL and chronic lymphocytic leukemia (CLL).[47]

***1.3.4.1 Molecular properties of concentrative nucleoside transporters.*** Due to the lack of specific inhibitors the characterization of sodium dependent nucleoside transport systems has been slow. The first successful cloning of transporter rCNT1was performed in 1994.[48] When expressed in *Xenopus* oocytes, rCNT1 demonstrated a high level of nucleoside transporter activity with substrate selectivity for pyrimidine nucleosides and adenosine. The human orthologue, hCNT1, was found to be 83% identical to rCNT1 in amino acid sequence and showed the transport characteristics of $Na^+$ dependent nucleoside transport and with selectivity for pyrimidine nucleosides (system *cit*). Both hCNT1 and rCNT1 contain 650 and 648 residues (71 kDa) respectively. The mammalian CNT proteins are predicted to have membrane topologies similar to that of rCNT1, which is shown to consist of 13 transmembrane domains with a large putatively extracellular glycosylated C terminus.[49]

The rCNT2 was first isolated from rat intestine[50] and has 659 amino acids with 14 transmembrane spanning regions and is 64% identical to rCNT1. The hCNT2 transports purine nucleosides and uridine, [51] thus mediating *cif* transport and is 83% identical to rCNT2. The mammalian CNT3 proteins (hCNT3) transport a broad range of nucleosides and thus mediate the *cib* process. The hCNT3 is 48 and 47% identical, respectively to hCNT1 and hCNT2. The predicted topology of hCNT3 is similar to that of h/rCNT1/2.[50]

**1.4 Regulation of nucleoside transport**

During the last few years, there has been increasing evidence that nucleoside transport expression may be correlated to rates of cellular proliferation.[21] In one study it was observed that v-*fps,* a transforming protein tyrosine kinase, induced a two fold increase in the nucleoside transport by rat-2 v-*fps* transformed fibroblasts.[52] The expression of *es* in hematopoeitic cells[23] has been shown to be stimulated when the cells are undergoing rapid cell division. However, the correlation of *es* transporter abundance with proliferation rates in the myeloblasts from AML patients has been controversial. In a study conducted on freshly isolated blasts from patients with AML and non-Hodgkin's lymphoma, it was observed that the number of transporters in these blasts correlated with proliferative rate.[53] It was also noted that the lymphomas had highest dependence of NBMPR site density on proliferative rate and the highest values were detected in patients with high-grade tumors (Burkitts lymphoma, early T-lymphoblastic lymphoma). However, in another study no correlation was observed between the *es* content and degree of proliferation of leukemia cells isolated from pre-treatment bone marrow samples from patients with AML.[54] Belt *et al.* pointed out that there were no methodological differences to explain the discrepancy between the two studies.[55] The

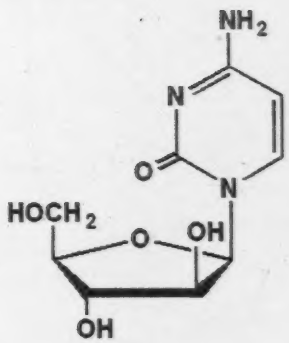
differences could be due to the difference in the nucleoside transport activity between myeloblasts found in peripheral blood and bone marrow.

**1.5 Cytarabine (araC)**

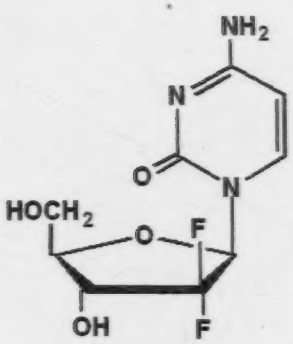
AraC is an antimetabolite nucleoside drug, which has been a mainstay in the treatment of leukemia since its introduction in the early 60's.[56] AraC is the most effective antimetabolite drug that is used in both induction and consolidation therapy of AML.[57] It is also employed in the therapy of ALL[13] and diffuse large cell lymphoma.[57] AraC is also included in the therapy of MDS, which frequently progress to AML[4].

***1.5.1 Mechanism of action.*** The transport of araC, (Figure 3) a nucleoside drug, across the cell membrane is a determinant of its cytotoxic activity.[16] The entry of araC into cells is a transporter-mediated process. A correlation between the number of transporter sites on leukemic cells and the formation of the cytotoxic metabolite of araC (araCTP) has been demonstrated.[15] Intracellularly, araC is converted to araCTP by the sequential action of deoxycytidine kinase (dCK), deoxycytidine monophosphate kinase (dCMP kinase) and nucleotide diphosphate kinase (NDP kinase).[57] However, this formation of the cytotoxic metabolite of araC is hindered by either the conversion of araC to inactive arabinosyl uracil (araU) by the enzyme cytidine deaminase or the conversion of arabinosylcytosine monophosphate (araCMP) to arabinosyluracil monophosphate (araUMP) by the enzyme dCMP deaminase.

AraCTP, is a DNA polymerase inhibitor that inhibits the binding of the enzyme to its normal substrate, deoxycytidine-5′-triphosphate (dCTP).[58] Another important cytotoxic effect of araC is its incorporation into deoxyribonucleic acid (DNA).[59] Thereafter, the incorporated araC causes inhibition of template function and chain




Cytarabine
Cytosine arabinoside (araC)
*(Cytosar-U)*




Gemcitabine
2'.2'-difluoro-2'-deoxycytidine (dFdC)
*(Gemzar)*

**Figure 3: Structures of nucleoside analogs: araC and dFdC.**

elongation.[60, 61, 62] The cytotoxicity of araC is cell cycle-phase dependent and it has greatest cytotoxic activity in the S-phase of the cell cycle.[63] AraC is not effective orally because of its rapid deamination in the liver and gastrointestinal tract (GIT) by cytidine deaminase to araU. AraC is generally administered as continuous intravenous infusion of 100-200 mg/m$^2$/day for 7-10 days resulting in peak plasma levels of 0.06-0.8 μM.[64]

***1.5.2 Combination therapy with araC.*** AraC is combined with another class of drugs in the induction therapy of AML. The usually employed induction therapy for AML is based upon the combination of araC and an anthracycline, most frequently daunorubicin[57], yielding remission rates of 55-70%.[65] Nowadays, a newer anthracycline, idarubicin, is often employed in the combination therapy of AML.[66, 67, 68] Idarubicin, is lipophilic compound and has a cytotoxic active metabolite, idarubicinol, with a prolonged plasma half life.[57] Idarubicin is now believed to be a potent antileukemic drug and is included in the regimens for AML along with araC.[69] Other chemotherapeutic agents such as thioguanine[70] and etoposide[68] have also been exploited in combination chemotherapy of AML and have resulted in complete remission rates of 55-83%.[71] Mitoxantrone, a synthetic anthracenedione derivative is also employed in some combination studies with araC.[72, 73] Myelosuppression and gastrointestinal epithelial injury are major side effects of araC. Gastrointestinal symptoms such as nausea, vomiting and diarrhoea are frequent during the period of administration.

***1.5.3 AraC transport.*** The uptake of most nucleoside analogs such as araC into cells is mediated by nucleoside-specific transporters present in the plasma membrane.[20, 21] At concentrations below 1 μM the rate of araC uptake was governed by membrane transport

but at concentrations greater than 10 μM, phosphorylation was the principal determinant of the uptake rate, in leukemic cells from patients.[17]

The *es* nucleoside transporter mediates the uptake of nucleoside analogs in hematopoietic cells.[20] AraC is a substrate of this *es* and *ei* nucleoside transporters.[4] In a study conducted on fresh leukemia cells from patients it was observed that NBMPR only partly protected the cells from araC cytotoxicity, thereby implying that the other nucleoside transporters such as *ei* or concentrative transporters might be contributing to araC uptake in the cells.[74] It was also shown that araC sensitivity and *es* nucleoside transporter abundance were correlated, indicating that *es* nucleoside transport was the major factor in araC uptake and activation.[74] In a recent study it was observed that araC is a substrate for the equilibrative nucleoside transporters, hENT1(*es*) and hENT2 (*ei*), but not for the concentrative nucleoside transporters, hCNT1 (*cit*) and hCNT3 (*cib*).[75] In a study conducted on acute leukemia cells from patients with araC, it was observed that *es* nucleoside transporter played a major role in sensitivity to araC.[16] A correlation between the number of transporter sites and formation of araCTP has also been established.[15] Also, a correlation between the *es* nucleoside transporter abundance and influx of araC has been demonstrated in leukemic blasts from patients.[29] It was shown that the araC influx and the number of NBMPR binding sites were correlated closely in the leukemic cells from patients and low inward flux of araC or few NBMPR binding sites were observed in a subset of acute leukemia patients who failed the induction therapy with araC.[29] Therefore, all these studies cumulatively affirm the correlation between araC cytotoxicity and *es* nucleoside transporter abundance in fresh leukemic blasts from patients.

***1.5.4 Resistance to araC.*** Despite the use of araC in the treatment of acute leukemia during the past few decades, failure to achieve clinical remission remains a major problem.[74] Although a number of putative mechanisms of araC resistance have been proposed, none of them have been established as a basis of clinical resistance in acute leukemia. AraC is hydrophilic in nature and nucleoside transporter mediated uptake is the major route of its entry into cells.[16] Therefore insufficient uptake might be a potential mechanism of araC resistance. A number of studies have demonstrated that deficiency in nucleoside transport is a possible mechanism of resistance to nucleoside analogs in cultured cell lines.[19, 29] In a study conducted by Galmarini *et al.* it was observed that the expression of *es* was related to the clinical response to araC.[19] The *es* nucleoside transporter abundance has been found to be correlated with the sensitivity to araC.[16] Thus, all these studies illustrate the role the membrane transport in araC resistance in leukemia, however it has not been established as a clinical basis of resistance.

One of the other common mechanisms of resistance proposed is a decrease in dCK activity.[76] This enzyme (dCK) plays a major role in the formation of araCTP; therefore variations in its activity may contribute to varied responses with araC chemotherapy. In a study performed in cells from patients with different acute leukemias, it was concluded that the variations in the amount of dCK did not account for the differences in the araCTP formation in different leukemias.[15] Therefore, the correlation between dCK and sensitivity to araC remains controversial.

Another enzyme, which plays a key role in araC metabolism and might account for araC resistance is cytidine deaminase.[57] Some studies have demonstrated that a reduced intracellular concentration of araCTP, the active metabolite of araC is a factor associated

with clinical response to araC.[59] Other proposed mechanisms of araC resistance include an increase in dCTP pools and increased levels of deoxycytidylate deaminase, as the deamination of araCMP may impede the accumulation of araCTP.[57] However although a number of factors have been indicated in resistance associated with araC, there still exists a lack of correlation of these factors with the clinical response to araC.

**1.6 Gemcitabine (dFdC)**

2′, 2′- Difluorodeoxycytidine (gemcitabine, *Gemzar*, dFdC) is a novel deoxycytidine analog with good activity against solid tumors.[77] It is widely employed in the therapy of advanced pancreatic cancer[78], non small cell lung cancer and transitional carcinoma of the bladder.[79, 80] Although dFdC has been shown to exert a good cytotoxic activity in human leukemic cell lines[77], its activity against leukemic cells *in vivo* still remains questionable.[81]

***1.6.1 Mechanism of action.*** A prerequisite for dFdC cytotoxicity is its transport across the cell membrane.[18, 82] It has been shown that dFdC is a substrate for four nucleoside transporters found in humans (*es*, *ei*, *cit* and *cib*).[18] The hENT1 (*es*) protein is ubiquitous and is the major means by which dFdC enters cultured human cells.[24] Intracellularly, dFdC is phosphorylated to difluorodeoxycytidine monophosphate (dFdCMP) by the enzyme dCK and further is converted to difluorodeoxycytidine diphosphate (dFdCDP) and difluorodeoxycytidine triphosphate (dFdCTP).[83] The metabolite of dFdC, dFdCTP, is the cytotoxic metabolite, which inhibits DNA synthesis.[84, 85] Also, dFdCDP is an inhibitor of ribonucleotide reductase, which causes depletion of deoxyribonucleotide pools required for DNA synthesis. However, the formation of dFdCDP and subsequently dFdCTP is hindered by the conversion of dFdC to difluorodeoxyuridine (dFdU) by the

enzyme cytidine deaminase.[57] It is known that dFdC is a cell cycle phase specific drug, which acts in the S phase of the cell cycle and also blocks the progression of cells through G1/S phase boundary.[77]

***1.6.2 Clinical activity of dFdC.*** The novel antimetabolite, dFdC is employed in the therapy of pancreatic cancer and is also included in first line regimens in non-small cell lung cancer.[86] In combination, it can be used with cisplatin for the treatment of bladder cancer[87] and non-small cell lung cancer. [79, 80, 86] A significant cytotoxic activity against human leukemia cell lines[77, 88] has also been demonstrated by dFdC. A number of studies have been conducted to explore the therapeutic potential of dFdC in patients with hematological malignancies.[81, 83, 89] The dose limiting toxicity of dFdC is myelosuppression. Other non-hematologic toxicities include fever, headache, diarrhea and stomatitis.[57, 90] Other acute toxicities include bronchospasm, cardiovascular problems such as myocardial infarction and arrythmia.[91] The most common method of dFdC infusion is a 30-minute infusion weekly for 3 weeks followed by one-week rest.[90]

***1.6.3 Resistance to dFdC.*** Although dFdC has been shown to exert a good cytotoxic activity in cell lines, its activity in hematological malignancies is not clinically significant. In a phase I clinical study conducted with dFdC in patients with relapsed or refractory acute leukemia or chronic myelogenous leukemia, it was observed that no patient achieved a complete remission.[81] The reduction in the percentage of the blasts in the marrow was disappointing. In another study conducted on 19 patients with relapsed (n=14) or primary refractory leukemia (n = 5), [83] all the patients had received araC-containing regimens in their initial therapy. However, none of the patients achieved a complete remission after treatment with dFdC.

**1.7 Research objectives**

Leukemia is a form of cancer characterized by abnormal proliferation of white blood cells. A number of anticancer nucleoside drugs are available for the treatment of leukemia. Despite the use of modern chemotherapy, clinical resistance is still a major problem associated with leukemia. Although a number of mechanisms of drug resistance have been proposed, none of them have been established as a basis of clinical resistance.

Nucleoside transporters present in the cell membrane are required for the uptake of araC and dFdC into cells. Studies have shown a correlation between the *es* nucleoside transporter abundance and araC influx.[29] A correlation between the *es* nucleoside transporter abundance and formation of the cytotoxic metabolite of araC, araCTP[15], has also been demonstrated. Recent studies have shown that there exists a correlation between the abundance of *es* nucleoside transporters and the cytotoxicity of nucleoside analogs such as araC[16] in leukemic blasts from patients. In another study it has been shown that *es* nucleoside transporter is a determinant of cytotoxicity of araC in blasts from pediatric patients with ALL.[92]

**In this thesis, it was hypothesized that *es* nucleoside transporter-mediated membrane flux is a determinant of the cytotoxicity of nucleoside drugs in leukemic blasts from patients**. With the aim of examining a correlation between nucleoside drug cytotoxicity and *es* nucleoside transporter function, that is, the *es* nucleoside transporter-mediated membrane flux, the present study evaluated the cytotoxicity and inward fluxes of araC and dFdC in cultured KG1 cells as well as in fresh leukemic blasts from patients with acute leukemia and MDS. In another part of this project, an *es* nucleoside transporter-deficient, dFdC-resistant cell subline was isolated from human T-lymphoblast

cell line, CEM/CCL1, which is partly *es*-deficient and resistant to araC. The isolation of such a subline would lend further support to the hypothesis that a deficiency in nucleoside transport may account for some instances of resistance to nucleoside analogs.

# Chapter 2

# MATERIALS AND METHODS

**2.1 Materials.** AraC, thymidine (dThd) and DPM were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ficoll-Paque was obtained from Amersham Biosciences, AB, Sweden. Cy-Chrome™ labeled anti CD45 was purchased from Becton Dickinson Immunocytometry Systems, San Jose, CA, USA. 2-CdA, NBMPR and SAENTA-fluorescein were prepared in this laboratory. Propidium iodide (PI) was purchased from Calbiochem, San Diego, CA, USA. [5-$^3$H]AraC (22 Ci/mmol), [5-$^3$H(N)]2′-deoxycytidine ([$^3$H]dCyd, 17 Ci/mmol) were obtained from Moravek Biochemicals, CA, USA. Eli Lilly and Company, Indianapolis, IN, USA provided [G-$^3$H]dFdC (23 Ci/mmol) and dFdC. Dilazep dihydrochloride was a gift from Hoffmann-La Roche & Co., Basel, Switzerland.

Transport oil was a mixture of light paraffin oil, J.T. Baker Inc., NJ, USA and Dow Corning 550 silicone oil, Dow Corning, Mississauga, ON, Canada, with a density of 1.03 g/ml. RNAse and dimethyl sulphoxide (DMSO, for cell culture, endotoxin and hybridoma tested, sterile filtered) were purchased from Sigma-Aldrich Co. Ltd., St. Louis, MO, USA. Fetal bovine serum (FBS) was from GIBCO™ Life Technologies, Ontario, Canada.

Ecolite and Triton X-100 were purchased from ICN Biomedicals Inc., Costa Mesa, CA, USA. Ion exchange papers were from Whatman International Ltd., Maidstone, Kent, UK. Roswell Park Memorial Institute (RPMI-1640) powdered medium was purchased from Sigma Chemical Co., St. Louis, MO, USA. Bio-Rad protein assay kit was from Bio-Rad Laboratories, Hercules, CA, USA.

**2.2 Preparation of nucleoside drug solutions.** *AraC solution.* AraC stock solution was prepared in double distilled water (dd$H_2O$). Required amount of araC was weighed and

dissolved in $ddH_2O$ in a polystyrene tube. The solution was then sterile filtered (in biological safety cabinet) into a sterile plastic snap cap tube. The stock solution was diluted in 0.01 M HCl and the concentration was determined ($\varepsilon_{281nm}$ = 13171) in a Beckman DU® 640 spectrophotometer. The stock solution was then diluted to the required concentrations in $ddH_20$ (secondary stock solutions). The stock and secondary stock solutions were stored at –20°C.

*DFdC, 2-CdA, dThd solutions.* DFdC, 2-CdA and dThd solutions were prepared in $ddH_2O$ from the dilutions of the respective sterile stock solutions, which were previously prepared in the laboratory. Both stock and secondary stock solutions (dFdC, 2-CdA and dThd) were stored at –20°C.

**2.3 Nucleoside transport inhibitor solutions**. *NBMPR solution.* Saturated solutions of NBMPR were made in 14 x 44 mm plastic screw cap vials in HSC buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$, pH 7.4). The vial was kept in the water bath (37°C) for 30 minutes, after which it was returned to room temperature and solution was filtered through a 0.22 μm millipore filter. The concentration of the solution was determined ($\varepsilon_{290\ nm}$ = 25,100) using a Beckman DU® 640 spectrophotometer. The NBMPR stock solution was then diluted to the required concentration (secondary stock solution) for the nucleoside flux or the cytotoxicity assays, just before use. Both the stock solution and the secondary stock solution were stored at room temperature (22°C).

*DPM Solution.* DPM stock solution was made in methanol (HPLC grade) and was stored at –20°C, protected from light. The stock solution was diluted in methanol (HPLC grade), filtered and the concentration was determined ($\varepsilon_{293\ nm}$ = 30,940) on a Beckman DU® 640 spectrophotometer. The stock solution was diluted to the required

concentration in sterile phosphate buffered saline (PBSA) of this composition: 137 mmol/l NaCl, 8.1 mmol/l $Na_2HPO_4.7H_2O$, 1.1 mmol/l $KH_2PO_4$ and 2.7 mmol/l KCl (pH 7.4) just before use. The stock solution was stored at –20°C.

*Dilazep Solution.* Dilazep hydrochloride stock solution was made in $ddH_2O$. The solution was filtered using a 0.22 μm millipore filter and its concentration determined ($\varepsilon_{266\ nm}$=18,420) using a Beckman DU® 640 spectrophotometer. The stock solution was then diluted to the required concentration (secondary stock solution) with $ddH_2O$. Both the stock solution and the secondary stock solution were stored at –20°C.

**2.4 Cell culture procedures.** All cell culture procedures and assays that involved cell growth were carried out in a biological safety cabinet. This project employed the human myeloblast cell line KG1[93] and human leukemic T-lymphoblast cell line, CCRF/CEM[94], which had been maintained in this laboratory over several years. The CEM/CCL1 subline was isolated from CCRF/CEM cells previously in this laboratory by using fluorescence activated cell sorting (FACS) to select the cells that were dimly stained with SAENTA-fluorescein, a tightly-bound ligand of the *es* nucleoside transporter. CEM/CCL1 cells were partly characterized and found to be partly *es* deficient at the time of their isolation. KG1, CCRF/CEM, CEM/CCL1, CEM/CCL1/G (dFdC selected CEM/CCL1 cells) and CEM/CCL1/Gw (CEM/CCL1/G cell cultures from which dFdC was withdrawn) cells were cultured in sterile 25 $cm^2$ flasks in RPMI 1640 medium containing 10% FBS and incubated in a humidified atmosphere of 5% $CO_2$ in air at 37°C. Cells were counted using a Coulter Counter (model $Z_F$), Coulter Electronics Inc., Hialeah, FL, USA. After determination of mean generation time (MGT), KG1 cells were sub-cultured twice a week and CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells

were subcultured three times a week to maintain the exponential growth of cells. The KG1 cells had a MGT of 30-35 hrs whereas the CCRF/CEM, CEM/CCL1 and CEM/CCL1/G cells had MGTs of 23-25 hr, 23-29 hr and 25-29 hr respectively. Cells used in the experiments were always taken from cultures that were undergoing exponential growth.

**2.5 Separation of leukemic cells from peripheral blood samples.** Peripheral blood samples were obtained from adult patients with AML, ALL or MDS with informed consent. The samples were collected in sodium heparin-containing vacutainer tubes and were kept at room temperature, 22°C for a maximum of 24 hr before use. The samples were diluted with sterile phosphate buffered saline A (PBSA) and the diluted blood was layered over a cushion of Ficoll-Paque for the separation of low-density cell fraction by density gradient centrifugation (IEC Centra MP4 Centrifuge, 400 x *g*, 30 min, 22°C). Not more than 22 ml of the cell suspension was layered over 15 ml Ficoll-Paque (with a ratio of 1.5:1 (v/v)) in a sterile 50-ml centrifuge tube. The low-density cell fraction was then collected from the Ficoll-Paque interface, washed and resuspended in PBSA. The cells were counted using a Coulter Counter, model $Z_F$ (Coulter Electronics, FL, USA) and then resuspended at a concentration of $1 \times 10^6$ cells/ml in RPMI 1640 medium with 2 mmol/l HEPES and 10% FBS (R/H/10F) for the cytotoxicity assay or at $2 \times 10^7$ cells/ml in RPMI$^t$ (RPMI 1640 medium with 25 mmol/l HEPES and 12 mmol/l NaCl, pH 7.4) for the nucleoside flux assay.

**2.6 Cytotoxicity assays.** Nucleoside drug cytotoxicity was assayed in human leukemia cell lines and in leukemic blasts from patients. For cell lines, cells were harvested centrifugally in a Damon/IEC division (CRU-5000) centrifuge (206 x *g*, 5 minutes),

22°C. After the centrifugation, the cells were washed and resuspended in R/H/10F at 1.0 x $10^5$ cells/ml. Leukemic blasts from patients were prepared in R/H/10F at 1 x $10^6$ cells/ml, as described in section 2.5. Briefly, 0.75 ml of the cell suspension was added to 0.75 ml of the culture medium containing graded concentrations of drug(s), with or without NBMPR in 24 well microculture plates. Two replicates were prepared for each drug concentration and cells were exposed to 1 μM NBMPR in two of the control wells to examine the effect of NBMPR on the cell viability as compared to the other two control wells, which lacked any drug. The plates were incubated in humidified atmosphere of 5% $CO_2$ at 37°C for 96 hr. After the incubation, the cells were centrifuged in an Eppendorf Model 5415 microcentrifuge (238 x *g*, 5 min), washed and resuspended in HSC buffer. The cell viability was then determined by flow cytometry. In cytotoxicity assays involving leukemic blasts from patient samples, cells were washed and resuspended in HSC buffer as the cultured cells described above. Surviving cells were stained with Cy-Chrome™ labeled anti-CD45, to identify and enumerate leukemic blasts in control and drug-treated samples.

The light scatter signals from these cells were then collected using a FACScan flow cytometer [Becton Dickinson Immunocytometry systems (BDIS), San Jose, CA, USA], allowing enumeration of cells surviving at each drug concentration. Cell viabilities (Total Events) were expressed as percentages of those in controls, which had no drug (for –NBMPR conditions) or which had 1 μM NBMPR (for +NBMPR conditions).

In order to determine the concentration dependence of protection by nucleoside transport inhibitors against araC cytotoxicity in KG1 cells, the cytotoxicity assay described above was modified. The KG1 cells were exposed to graded concentrations of

transport inhibitor (NBMPR or dilazep) ± araC (50 nM) for 96 hr. The controls for the +araC conditions (in duplicate) contained 50 nM araC but lacked transport inhibitor whereas the controls for –araC conditions (in duplicate) contained no drug. After the incubation period, the cells were harvested and resuspended in HSC buffer as described above.

The signals from the cells were detected and analyzed using a FACScan flow cytometer [Becton Dickinson Immunocytometry systems (BDIS), San Jose, CA, USA] and CellQuest™ software. Cell viabilities were expressed as percentage of those in control wells. GraphPad Prism® software was used to fit the nonlinear regression curves to the plots of cell viability versus the log of drug concentration.

**2.7 Nucleoside transport experiments.** All the nucleoside influx experiments were carried out at room temperature, 22°C, in a biological safety cabinet. Cells were suspended in RPMI$^t$ medium for the influx experiments at 2 x $10^7$ cells/ml. The leukemic blasts were isolated from peripheral blood of samples as described in section 2.5 and resuspended in RPMI$^t$ at the concentration of 2 x $10^7$ cells/ml. An oil stop centrifugation method was used to measure the inward fluxes of the $^3$H-labeled nucleoside permeants. Permeant solutions containing $^3$H-nucleoside and non-radioactive nucleoside (1.6 μM, 2x concentration), with or without the nucleoside transport inhibitor, NBMPR were employed. In this method, 100 μl of cell suspension (cultured cells or patient samples) was layered over the transport oil in a microfuge tube (with cut off cap). The assay was initiated by adding 100 μl of $^3$H-permeant solution, with or without the transport inhibitor to the cell suspension. After a time period of 5-300 sec, the mixture was centrifuged (30 sec, 11,700 x *g*) in an Eppendorf Model 5414 microcentrifuge. Since it takes about 2 sec

for the cells to pass through oil[95], the nucleoside uptake continues during the cell pelleting, effectively adding a 2-sec time interval to the exposure time in the assay.[95] During this centrifugation step the cells were pelleted under the oil, ending uptake of the nucleoside permeant into the cells from the permeant solution. The cells, being denser than the oil were pelleted to the bottom of the tube and the radioactive permeant solution remained above the oil. The radioactive permeant solution was then aspirated and 1.0 ml $ddH_2O$ was added to the tube to wash any radioactivity remaining (above the oil) on the wall of the tube. Finally, $ddH_2O$ was aspirated along with the transport oil, without disturbing the cell pellet and the cells were lysed overnight in the capped tubes using 200 µl of 5% Triton X-100. The microfuge tubes were vortexed, opened and transferred along with their cut off caps to scintillation vials together with 10 ml of Ecolite scintillation fluid (ICN, Costa Mesa, CA, USA). Two blank samples were also prepared to determine the background radioactivity. Radioactive standards for the determination of specific activity (in triplicate) were prepared by exposing the cells to RPMI[t] medium and pelleting under the oil as described above. $^3$H-permeant (2x) solutions (20 µl) were added to the cells after the cell lysis with Triton X-100 and counted for radioactivity. The radioactivity of each vial was determined by liquid scintillation counting and cellular content of $^3$H-permeant calculated as follows:

$$\text{pmol } ^3\text{H-permeant}/10^6 \text{ cells} = \frac{\text{cpm/pellet (average of triplicates – background)}}{\text{specific activity (cpm/pmol) x 2 x } 10^6 \text{ cells/pellet}}$$

Where, cpm represents counts per minute measured in each scintillation vial. Specific activity was calculated as follows:

$$\text{Specific activity (cpm/pmol)} = \frac{\text{cpm/pellet (average of standards – background)}}{20\ \mu l \times 1.6\ \text{pmol}/\mu l}$$

A replicate value was considered as an outlier if its value exceeded the mean of the other two replicates and was not used in the data reported.

*2.7.1 Concentration dependence of araC uptake in a human leukemic myeloblast cell line (KG1 cells).* Initial rates of araC uptake, which were measures of inward fluxes, were determined as the difference between araC uptake after 5 and 10 seconds of exposure of cells to graded concentrations of the permeant. The initial rate of $^3$H-permeant uptake was measured as described in section 2.5 and plotted as a function of araC concentration. Briefly, KG1 cells were exposed to permeant solutions containing graded concentrations of araC both in the presence and absence of NBMPR. Nonlinear regression analysis of the data was performed (using GraphPad Prism, Version 3.02) and the kinetic constants $K_m$ and $V_{max}$ were determined using Michaelis-Menten equation: -

$$v = \frac{V_{max} \cdot S}{K_m + S}$$

Wherein, $V_{max}$ represents the maximum inward flux, $K_m$ is the Michaelis-Menten constant, S is the nucleoside permeant concentration and v is the inward flux at a particular permeant concentration.

**2.8 Selection of dFdC resistant (CEM/CCL1/G) cells.** CEM/CCL1/G cells were isolated from CEM/CCL1 cells, which were previously isolated in this laboratory.

CEM/CCL1 cells were cultured in the presence of stepwise increasing concentrations of dFdC in the culture medium (RPMI with 10% FBS) for isolating the CEM/CCL1/G cells. In the selection process, CEM/CCL1 cells were counted using a Coulter counter and after determination of the cell counts, the required amount of medium was added to the 25 $cm^2$ flasks. The drug solution (dFdC solution in $ddH_2O$) was added as a bead on the flask wall, just above the medium and mixed gently, before the addition of the cells. The addition of dFdC solution to the medium before addition of the cells ensured homogeneous exposure of cells to dFdC in the medium. The culture medium (20 ml) contained 21.3 µl of 9.4 µM dFdC in $ddH_2O$, (10 nM of dFdC) in the beginning and the concentration was increased in a stepwise manner i.e. 10, 20, 50, 100, 200, 500 nM once stable growth rates were achieved. The cells were incubated in humidified atmosphere at 37°C, 5% $CO_2$. On exposure to dFdC, only the resistant cell population proliferated while the sensitive population could not survive. The selection procedure occurred over 2 months and yielded a dFdC-resistant subline, CEM/CCL1/G.

**2.9 Determination of *es* nucleoside transporter content.** The cellular abundance of *es* nucleoside transporters was determined by employing the fluorescent *es* nucleoside transporter ligand, SAENTA-fluorescein, in a flow cytometry procedure. Cells (1 x $10^5$) were harvested centrifugally in an Eppendorf Model 5415 microcentrifuge (238 x *g*, 5 min, 22°C), washed free of the medium and resuspended in HSC buffer for determination of total binding of SAENTA-fluorescein or in HSC buffer containing 5 µM NBMPR for measuring nonspecific binding. Prior treatment of the cells with NBMPR for 30 min, 22°C prevents site-specific binding of SAENTA-fluorescein to the *es* transporter sites. To measure total binding, the cells were exposed to SAENTA-fluorescein for at least 15

minutes at 22°C. Non-specific binding of cells was determined by adding SAENTA-fluorescein solutions that contained NBMPR to the NBMPR-treated cells. The cell suspensions were protected from light during this 15-minute incubation period.

Cell associated fluorescence and light scatter signals were detected with a FACScan flow cytometer [Becton Dickinson Immunocytometry systems (BDIS), San Jose, CA, USA]. SAENTA-fluorescein molecules were excited with 488 nm light from an argon laser and the cell associated emitted fluorescence was collected at 530 nm (FL1).

The fluorescence signals from 10,000 cells were acquired and analyzed using Cell Quest software (Becton Dickinson Immunocytometry Systems). The mean relative fluorescence intensity (RFI) values from the histograms of SAENTA-fluorescein fluorescence was a measure of SAENTA-fluorescein bound to the cells. The *es* nucleoside transporter-specific binding was calculated as the difference between the total and nonspecific binding.

**2.10 Cell cycle phase distribution measurements.** The cell cycle phase distribution of cultured human leukemic cells was determined by using the DNA stain, PI. Cells (2 x $10^6$) were harvested from exponentially proliferating cultures in a Damon/IEC division (CRU-5000) centrifuge at 206 x *g*, 5 min, washed in 10 ml PBSA and resuspended in 0.80 ml PBSA. The cells were counted using Coulter counter and after counting, 1.9 ml of cold 70% ethanol was added drop wise with gentle mixing and the cells were stored at 4°C overnight or longer.

For the staining, the cells were washed free of ethanol (centrifuged at 18 x *g*, 5 min, 22°C), resuspended in PBSA (left for 60 sec) and centrifuged again (18 x *g*, 5 min). The cell pellet was resuspended in a working solution of 1 ml 0.1% Triton-X 100 in PBSA, 2

mg RNase A and 200 µl PI solution (1 mg/ml in PBSA). The cell suspensions were protected from light, at 22°C. The cell suspensions were then filtered through a nylon mesh (50 µm) and analyzed by flow cytometry using a FACScan flow cytometer [Becton Dickinson Immunocytometry systems (BDIS), San Jose, CA, USA] within 30 min. The results were analyzed using ModFit LT 2.0 software [Becton Dickinson Immunocytometry systems (BDIS), San Jose, CA, USA].

**2.11 Deoxycytidine kinase assay.** The activity of dCK was determined in CCRF/CEM, CEM/CCL1 and CEM/CCL1/G cells. Cells (6 x $10^6$) were harvested centrifugally in a Damon/IEC division (CRU-5000) centrifuge (206 x *g*, 5 min, 22°C), washed and resuspended in PBSA (with cell concentration about 1 x $10^6$ cells/ml). After the resuspension, cell counts were determined using a Coulter counter, cells were pelleted (206 x *g*, 5 min, 22°C) and supernatant was discarded. The cell pellet was resuspended in PBSA, transferred to a clean microfuge tube and centrifuged at 11,700 x *g*, 30sec and 22°C in an Eppendorf Model 5415 centrifuge. The supernatant was discarded and cells were resuspended in homogenization buffer containing 50 mmol/l Tris-Cl, 1 mmol/l dithiothreitol (DTT) and 0.2 mmol/l phenylmethylsulphonylfluoride (PMSF), pH 7.6 and homogenized using a polypropylene pellet pestle. The homogenized cells were centrifuged at 11,700 x *g*, 10 min and 4-8°C in an Eppendorf Model 5415 centrifuge and the cell extract was transferred to another microfuge tube. Assay tubes were prepared containing 1.0 µM [$^3$H]dCyd, 1mmol/l DTT, 5 mmol/l $MgCl_2$ and 15 mmol/l sodium fluoride (NaF) in 50 mmol/l Tris-Cl ( pH 7.6), in a total volume of 200 µl.

Also, anion exchange discs, DE81 (Whatman® ion exchange paper circles, Whatman International Ltd., Maidstone, England) were prepared, which would bind the

$^{3}$H-nucleotide metabolites that would be formed by the reactions catalyzed by cell extracts. The anion exchange discs included substrate control discs, unwashed control discs, time zero discs, and 15-minute time point discs. Two blank discs were also prepared to measure background radioactivity.

The assay tubes, were equilibrated in water bath (37°C) for 5 minutes. Also, measured portions (20 µl) of the cell extract were transferred to a clean microfuge tube for protein determination (discussed later).

The kinase reaction was started by addition of 20 µl cell extract (equivalent to about $6 \times 10^5$ cells) to the assay tubes. The timer was started and after the incubation times (0 minute or 15 minutes), samples of the assay mixtures (50 µl) were spotted on the prelabelled discs. The spotting of the assay mixtures on the discs terminated the reaction.

After all the assay mixtures had been spotted, the discs were dried at room temperature (10 min) and washed (except the unwashed controls) in 1.5 liters of 5 mmol/l of ammonium formate solution. The solution was stirred gently (10-20 min) as the discs were very fragile and was replaced 3 times with fresh batches of ammonium formate solution.

The discs (both washed and unwashed) were then transferred to scintillation vials together with 1.0 ml of 0.2 M KCl/0.1 M HCl solution for at least 15 minutes before the addition of 10 ml of Ecolite scintillation fluid (ICN, Costa Mesa, CA, USA). The samples were read for radioactivity in a liquid scintillation counter.

**2.12 Protein determination in CCRF/CEM, CEM/CCL1 and CCL1/G cells.** Protein content of the CCRF/CEM, CEM/CCL1 and CEM/CCL1/G cell extracts was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The

Bio-Rad protein assay was based upon the method of Bradford.[96] The Bio-Rad dye-binding assay is an assay based on the differential color change of Coomassie blue dye in response to change in protein concentration. Bovine serum albumin was used as standard to create the standard curve for protein determination.

**2.13 Statistical Analysis.** Student's t-test was used to measure statistical significance ($p < 0.05$). The correlation between the *es*-mediated nucleoside influx and cytotoxicity was determined using a Pearson correlation test by GraphPad Prism 3.02. The Tukey-Kramer multiple comparison test was used to compare the PI staining, SAENTA-fluorescein staining and deoxycytidine kinase assay parameters between the CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells. The data are represented as mean ± SD except for the $IC_{50}$ values, which are represented as mean values (95% confidence intervals). Statistical test for the *es* transporter abundance and deoxycytidine kinase assay was performed with the level of significance at $p < 0.0001$.

**2.14 Outliers.** Outliers from three different experiments were not included in the data. In one of the nucleoside flux experiments, with sample no.15 one vial was not counted by the liquid scintillation counter because of inappropriate dimension of the vial. In another two inward flux experiments, with sample no. 3 and sample no. 41 one of the values(s) was approximately 3.6 and 5.4 times higher respectively than the mean of other two values and were not included in the final data.

# Chapter 3

# RESULTS

**3.1 Cytotoxicity and inward flux measurements of araC in the human leukemic myeloblast cell line, KG1**

***3.1.1 Cytotoxic effects of araC and modulation of araC cytotoxicity by NBMPR in KG1 cells.*** This project examined the role of membrane transport in the sensitivity of leukemia cells from patients to nucleoside drugs. As the majority of clinical samples were from patients with AML, KG1 leukemic myeloblasts were chosen as a reference for comparing the results obtained from a variety of clinical samples. To begin, the KG1 cells were characterized with respect to *es* nucleoside transport activity and sensitivity to araC and dFdC. The cytotoxicity of araC was evaluated and was pharmacologically manipulated by use of the nucleoside transport inhibitor, NBMPR, as shown in Figure 4. The $IC_{50}$ value for araC after exposure of cells to the nucleoside for 96 hours was 6.8 (5.2-8.8) nM. NBMPR at a concentration of 1 μM blocked *es* transporter sites and significantly protected the cells from the cytotoxicity of araC, yielding an $IC_{50}$ value of 32 (21-48) nM (Figure 4). The $IC_{50}$ values were statistically different from each other ($p < 0.05$). The effects of other *es* nucleoside transport inhibitors on araC cytotoxicity were also examined in KG1 cells. Both dilazep and DPM were tested at 1 μM concentrations. As shown in Table 1 both transport inhibitors protected the cells from the cytotoxic effects of araC, with $IC_{50}$ values of 34 (25-45) nM and 100 (70-150) nM respectively (Figure 4). Protection against araC cytotoxicity by DPM was statistically significant, whereas protection by dilazep was not.

***3.1.2 Concentration dependence of protection by nucleoside transport inhibitors against araC cytotoxicity in KG1 cells.*** These experiments were performed with the aim of evaluating the concentration dependence of the effects on araC cytotoxicity of

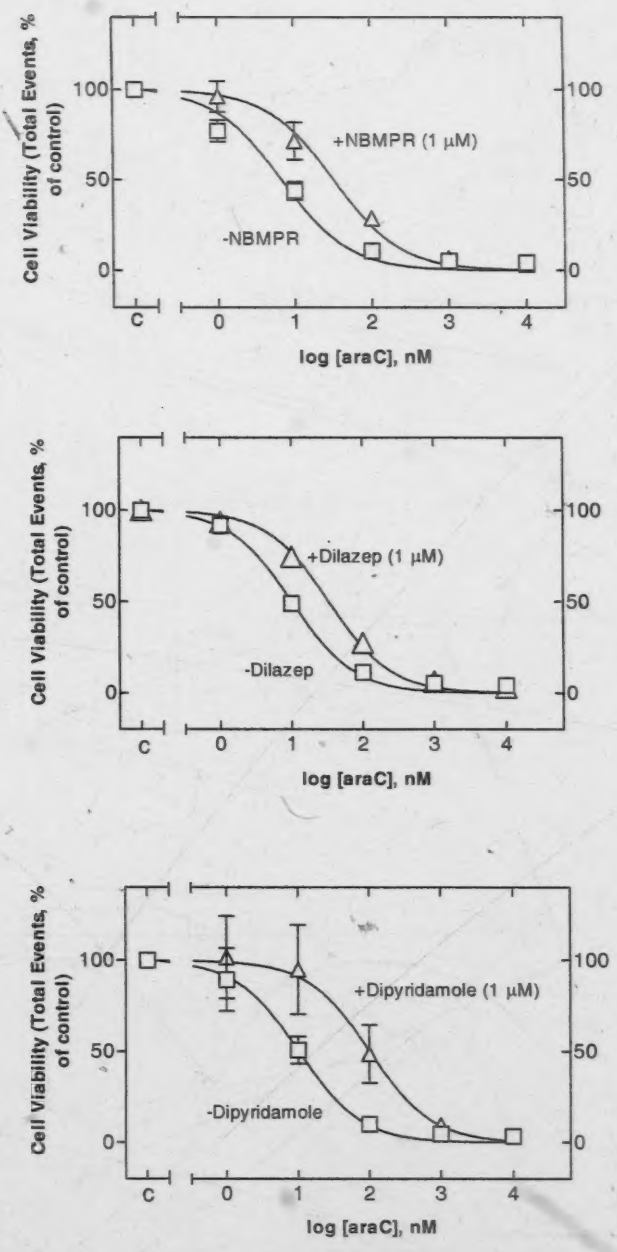



Figure 4: Concentration-effect curves for araC cytotoxicity in the absence and presence of transport inhibitors in KG1 cells. Cells were cultured in medium containing graded concentrations of araC in the absence (□) or presence (Δ) of 1 μM NBMPR. After 96 hours, the cell viability was measured by flow cytometry. The data shown are the mean ± SD (n = 8). Data were pooled from four separate experiments, each performed in duplicate.

**Table 1: The effect of *es* nucleoside transport inhibitors in modulating the cytotoxicity of araC.** The $IC_{50}$ values were found to be statistically significantly higher ($p < 0.05$) in the presence of NBMPR or DPM. The data shown are the mean (95 % confidence interval, n = 8). Data were pooled from four separate experiments, each performed in duplicate.

| Transport inhibitor (1 μM) | $IC_{50}$, araC (nM) -Inhibitor | $IC_{50}$, araC (nM) + Inhibitor |
|---|---|---|
| NBMPR | 6.8 (5.2-8.8) | 32 (21-48)* |
| Dilazep | 10 (7.6-13) | 34 (25-45) |
| DPM | 10 (8.4-12) | 100 (70-150)* |

* Statistically significant ($p < 0.05$).

nucleoside transport inhibition by NBMPR and dilazep in KG1 cells. It was also examined whether the transport inhibitors had any effect on cell viability in the absence of araC. Upon exposing the cells to graded concentrations of NBMPR, in the presence of araC (50 nM) it was observed that NBMPR protected the cells from cytotoxic effects of araC in a concentration dependent manner, with an $IC_{50}$ value of 19 (6.6-54) nM. It was also observed that NBMPR, alone, did not affect the cell viability (Figure 5). Similarly, dilazep did not have any effect on the cell viability on its own, but significantly protected the cells from the cytotoxic effects of araC in a concentration dependent manner (Figure 5). The $IC_{50}$ value for dilazep was found to be 12 (2.3-60) nM.

***3.1.3 [$^3$H]araC influx in KG1 cells.*** The initial rate of [$^3$H]araC uptake was determined, which was a measure of inward flux of the nucleoside analog. The initial rate of [$^3$H]araC uptake was measured in KG1 cells by employing the oil stop centrifugation technique at 22°C. The cells were exposed to permeant solutions containing [$^3$H]araC (0.8 $\mu$M) ± NBMPR (1 μM) for various time intervals (5-300 seconds). Figure 6 shows the time courses of [$^3$H]araC uptake in KG1 cells. The uptake of [$^3$H]araC was found to be linear up to 300 seconds with a flux of 14 ± 0.71 fmol [$^3$H]araC/$10^6$cells/sec. The *es* nucleoside transport inhibitor, NBMPR, at a concentration of 1 μM, significantly inhibited the flux of 0.8 μM araC in these cells, reducing it to 1.5 ± 0.13 fmol [$^3$H]araC/$10^6$cells/sec, indicating that *es* nucleoside transport was significantly contributing to [$^3$H]araC uptake in KG1 cells.

***3.1.4 Concentration dependence of araC influx in KG1 cells.*** Kinetic experiments were performed in which inward fluxes of [$^3$H]araC were measured in KG1 cells as a function

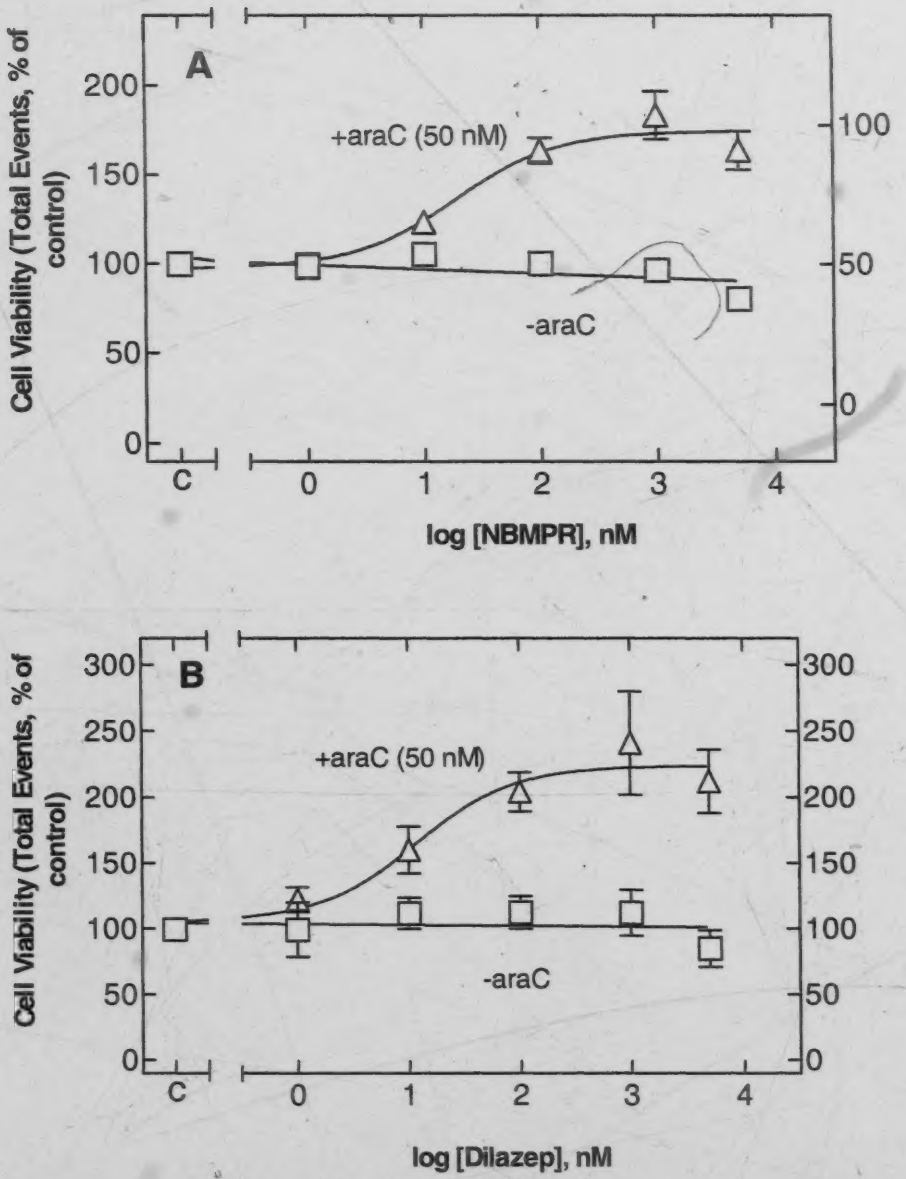



**Figure 5: Concentration dependence of the protection by *es* nucleoside transport inhibitors against araC cytotoxicity in KG1 cells.** Cells were exposed to graded concentrations of NBMPR (Panel A) or dilazep (Panel B), in the absence (□) or presence (Δ) of araC for 96 hours. After the incubation period, the cells were washed and cell viability was examined by flow cytometry. The $IC_{50}$ values for the protection by NBMPR and dilazep were 19 (6.6-54) nM and 12 (2.3-60) nM respectively. Data represented are mean ± SD (n = 8). Data were pooled from four separate experiments, each performed in duplicate.

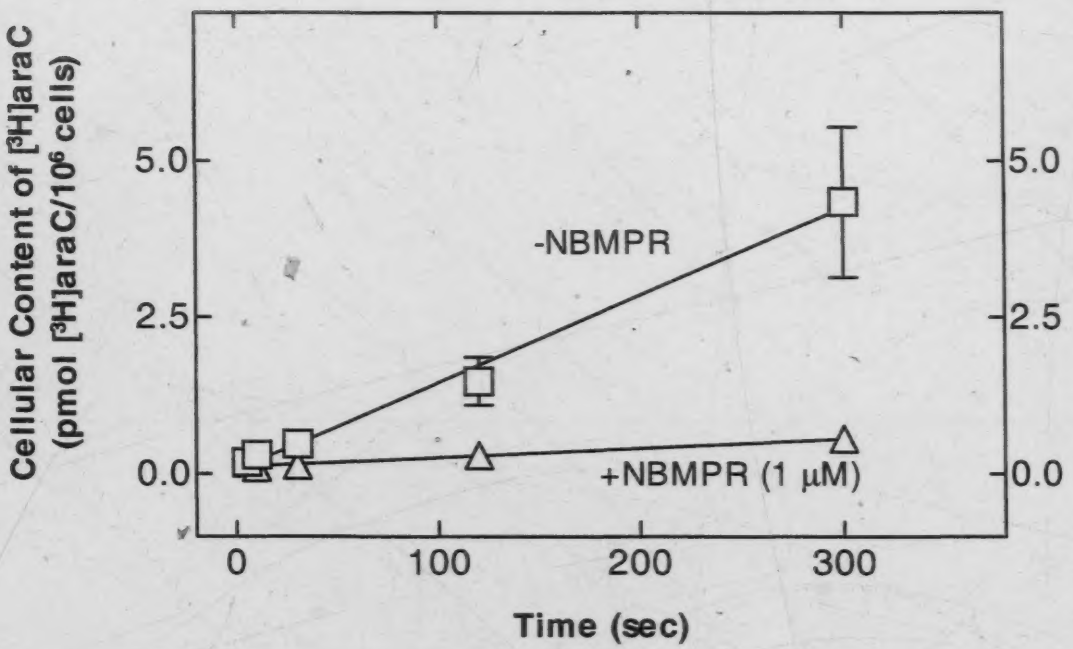



**Figure 6: Time courses of [$^3$H]araC uptake in KG1 cells and the effect of 1 μM NBMPR.** [$^3$H]araC inward fluxes were measured without inhibitor (□) and with 1 μM NBMPR (Δ). Data represent means ± SD (n = 18). Data were pooled from six separate experiments, each performed in triplicate.

of araC concentration at 22 °C. Initial rates of araC uptake, which were measures of inward fluxes, were determined as the difference between araC uptake after 5 and 10 seconds of exposure of cells to graded concentrations of the permeant. Figure 7 shows the saturability of [$^3$H]araC uptake in KG1 cells.

The kinetic constants $V_{max}$ and $K_m$ were 57 ± 4.2 pmol [$^3$H]araC/$10^6$ cells/sec/μM and 240 ± 53 μM, respectively. As seen in Figure 7, NBMPR (1 μM) completely inhibited the inward flux of [$^3$H]araC in KG1 cells at all araC concentrations tested. [$^3$H]AraC uptake in the presence of NBMPR was concentration independent, and found to be –0.050 ± 0.040 pmol [$^3$H]araC/$10^6$ cells/sec, a value that was not significantly different from zero.

***3.1.5 Concentration dependence of the inhibition of araC influx by NBMPR in KG1 cells.*** The initial rate of araC uptake was measured in KG1 cells exposed to solutions containing 0.8 μM [$^3$H]araC ± graded concentrations of NBMPR. The experiment of Figure 8 shows that the inhibition of araC influx in KG1 cells was concentration dependent. With increasing concentrations of NBMPR from 0-1 μM, a reduction in the inward flux of [$^3$H]araC was evident. The $IC_{50}$ value for NBMPR for inhibition of araC influx was found to be 12 (3.1-50) nM. This $IC_{50}$ value for NBMPR inhibiton of araC influx was statistically significantly different ($p < 0.05$) than the $IC_{50}$ value for protection by NBMPR against araC cytotoxicity in KG1 cells (19 (6.6-54) nM).

**3.2 Cytotoxicity and inward flux of dFdC in KG1 cells**

***3.2.1 Cytotoxic effects of dFdC and modulation of dFdC cytotoxicity by NBMPR in KG1 cells.*** The *in vitro* sensitivity of KG1 cells to another nucleoside analog, dFdC, and to dFdC/NBMPR combinations was studied. The assays were performed by flow

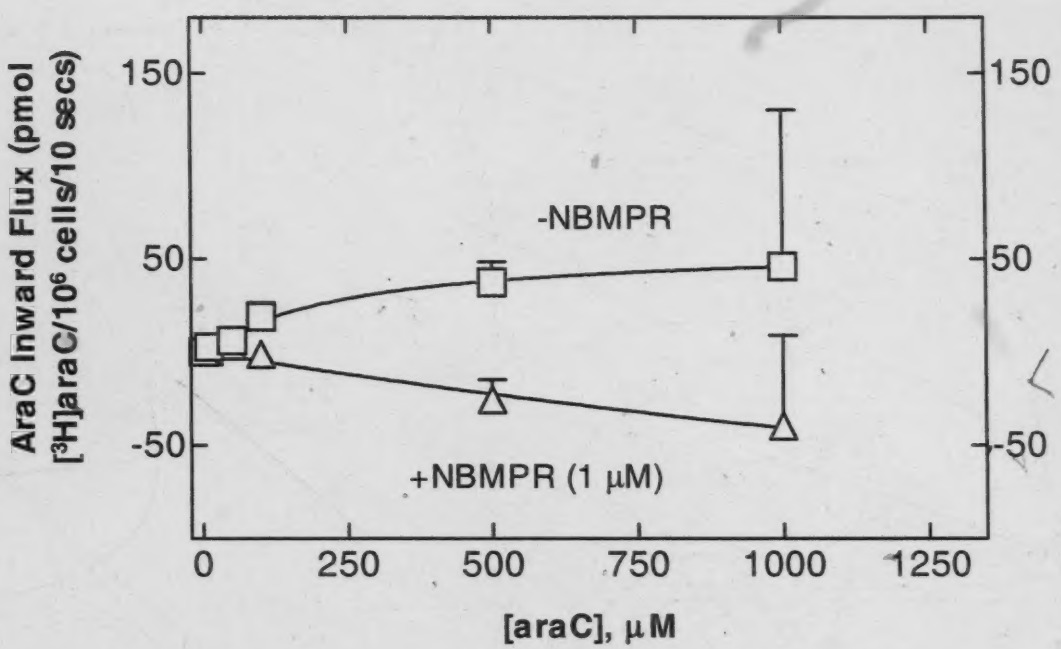



**Figure 7: Concentration dependence of araC influx in KG1 cells.** The initial rates of [$^3$H]araC uptake were plotted as a function of araC concentration in the absence (□) and presence (Δ) of NBMPR. Data represent means ± SD (n = 6). Data were pooled from two separate experiments, each performed in triplicate.

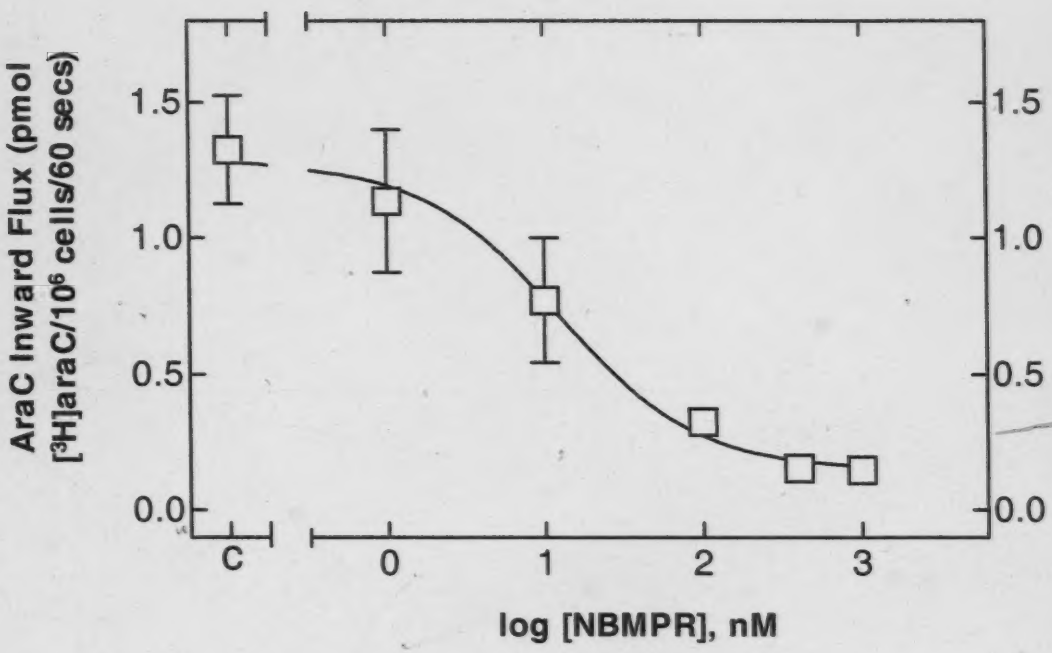



**Figure 8: Concentration dependence of the inhibition of araC influx by NBMPR in KG1 cells.** Cells were exposed to 0.8 μM [$^3$H]araC in the presence of graded concentrations of NBMPR. The $IC_{50}$ value for NBMPR for inhibition of araC influx was 12 (3.1-50) nM. The data represent mean ± SD (n = 9). Data were pooled from three separate experiments, each performed in triplicate.

cytometry. Concentration-effect curves were constructed to measure the cytotoxic effects of dFdC and to study the effects of the *es* nucleoside transport inhibitor, NBMPR, in modulating the cytotoxicity of dFdC. Figure 9 shows the cytotoxic effects of graded concentrations of dFdC in KG1 cells and the effects of 1 μM NBMPR on the cytotoxicity of dFdC. After 96 hours of exposure to dFdC, the $IC_{50}$ value for dFdC was 4.3 (2.8-6.7) nM. However, the $IC_{50}$ value in the presence of 1 μM NBMPR increased about 9-fold, 41 (21-78) nM. This increase indicates that NBMPR significantly protected the cells from the cytotoxic effects of dFdC.

***3.2.2 Inward flux of [$^3$H]dFdC in KG1 cells and the effect of the transport inhibitor, NBMPR.*** Time courses of [$^3$H]dFdC uptake in KG1 cells yielded a value for the initial rate of [$^3$H]dFdC uptake, which was a measure of the inward flux of [$^3$H]dFdC. Also, the *es* nucleoside transport inhibitor, NBMPR, was employed to identify the nucleoside transport process that accounted for dFdC uptake in these cells. Uptake of 0.8 μM [$^3$H]dFdC into the cells was measured during time intervals of 5-300 seconds by using the oil stop centrifugation technique. The uptake of [$^3$H]dFdC was found to be linear with time up to 300 seconds. The inward flux of [$^3$H]dFdC in these cells was 5.4 ± 0.70 fmol [$^3$H]dFdC/$10^6$cells/sec. NBMPR, at a concentration of 1 μM significantly inhibited the flux of 0.8 μM dFdC (Figure 10). The influx rate of [$^3$H]dFdC in the presence of 1 μM NBMPR was found to be 0.40 ± 0.33 fmol [$^3$H]dFdC/$10^6$ cells/sec, which was statistically different from the rate in the absence of NBMPR ($p < 0.0001$).

**3.3 Cytotoxicity and inward flux of araC in fresh leukemic blasts from patients**

***3.3.1 AraC cytotoxicity in fresh leukemic blasts from patients.*** The objective of this set of experiments was to observe the sensitivity of fresh leukemic blasts from patients to the

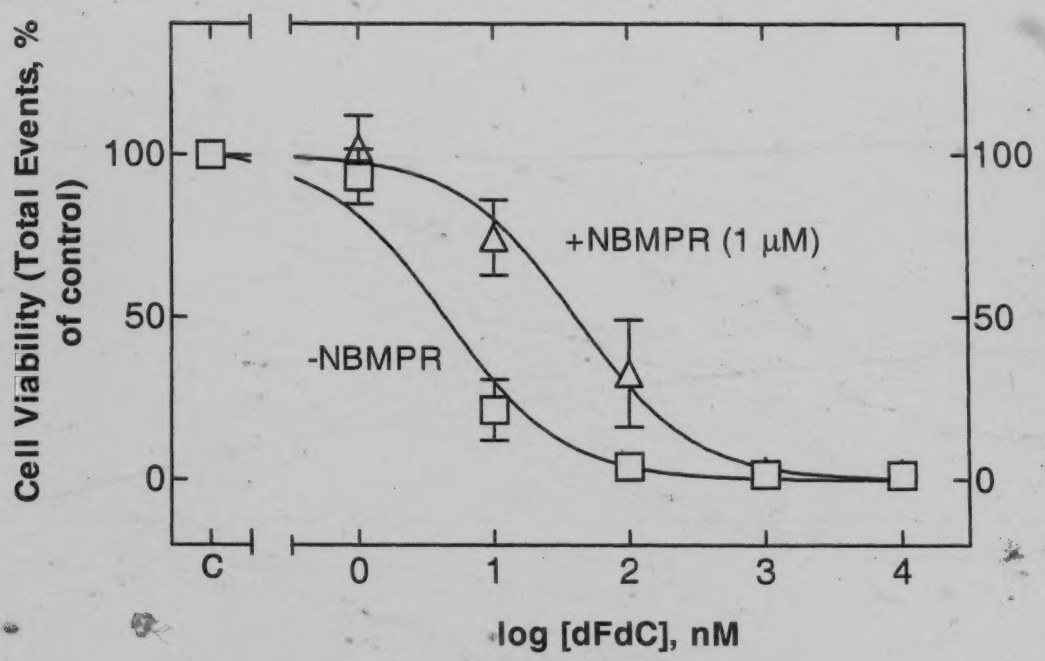



**Figure 9: Concentration effect curves for dFdC cytotoxicity in the absence and presence of NBMPR in KG1 cells.** The cells were cultured in medium containing graded concentrations of dFdC in the absence (□) or presence (Δ) of 1 μM NBMPR. After 96 hours the cell viability was measured by flow cytometry. The data shown are mean ± SD (n = 6). Data were pooled from three experiments, each performed in duplicate.

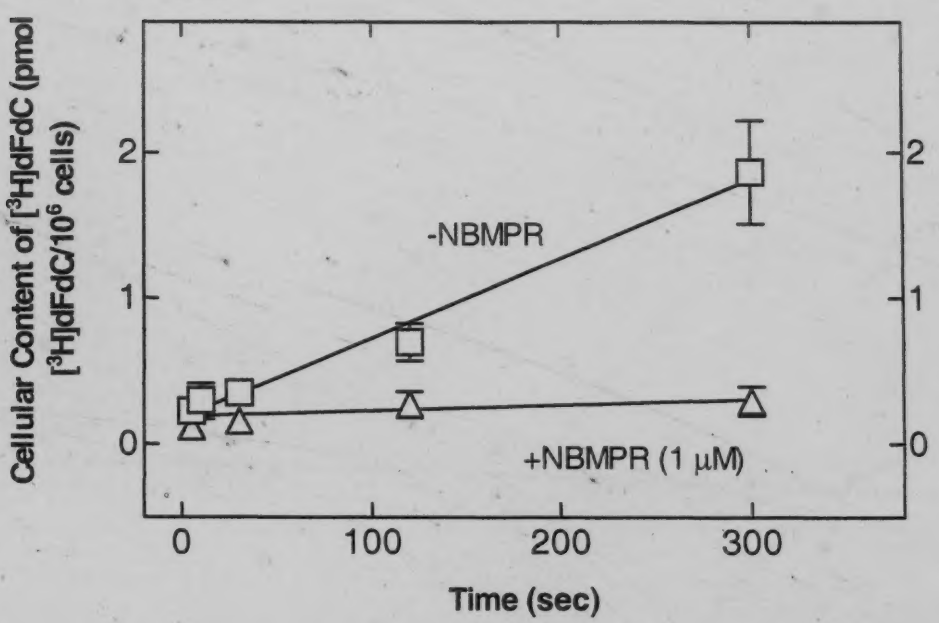



**Figure 10: Time courses of [$^3$H]dFdC uptake in KG1 cells and the effect of 1 μM NBMPR.** The uptake of 0.8 μM dFdC was measured in cells suspended in RPMI[t] medium, with (Δ) or without (□) 1 μM NBMPR. Uptake of 0.8 μM araC was measured at 22°C at the indicated time intervals. Data represent means ± SD (n = 9). Data were pooled from three experiments, with each experiment carried out in triplicate.

cytotoxic effects of araC. Leukemic blasts were isolated from peripheral blood samples by using a Ficoll-Paque centrifugation technique. After a 96-hr incubation period with araC ± NBMPR, the cells were harvested, stained with CyChrome™-labeled anti CD45 (Figure 11).

*In AML samples.* Samples of leukemic blasts from 20 patients with AML were analyzed. Table 2 illustrates the interpatient variability in sensitivity to the nucleoside analog, araC, with $IC_{50}$ values for araC cytotoxicity ranging from 0.05 – 4.6 μM, with a mean of 1.6 ± 0.4 μM (mean ± SEM, n = 19). The $IC_{50}$ values for these samples were 15 - 676 fold higher than the $IC_{50}$ value for araC cytotoxicity observed in KG1 cells ($IC_{50}$, 6.8 nM). In 1 of the 20 AML blast samples examined, the $IC_{50}$ value for araC was greater than 10 μM, which was the highest concentration tested.

Also, in these araC cytotoxicity experiments, effects of the blockade of *es* nucleoside transporter sites were measured by exposing the cells simultaneously to araC and to NBMPR at a concentration of 1 μM (Figure 12). The cells, in which the *es* nucleoside transporter sites were blocked by exposure to NBMPR, were protected from the cytotoxic effects of araC. In 11 of 20 AML patient samples NBMPR significantly protected the cells from the cytotoxic effects of araC, yielding an $IC_{50}$ value > 10 μM. In one of the sample (no. 40) the $IC_{50}$ values for araC cytotoxicity were > 10 μM, both in the absence and presence of NBMPR. In the other 8 of 20 AML samples, the $IC_{50}$ values in the presence of NBMPR ranged between 0.12 – 3.8 μM (Table 2) and NBMPR significantly ($p < 0.05$) protected the cells from cytotoxic effects of araC in 7 of these 8 samples. Protection by NBMPR was not statistically significant ($p < 0.05$) in sample No. 48. Thus, through the use of NBMPR as a transport inhibitor, *es* nucleoside transporter

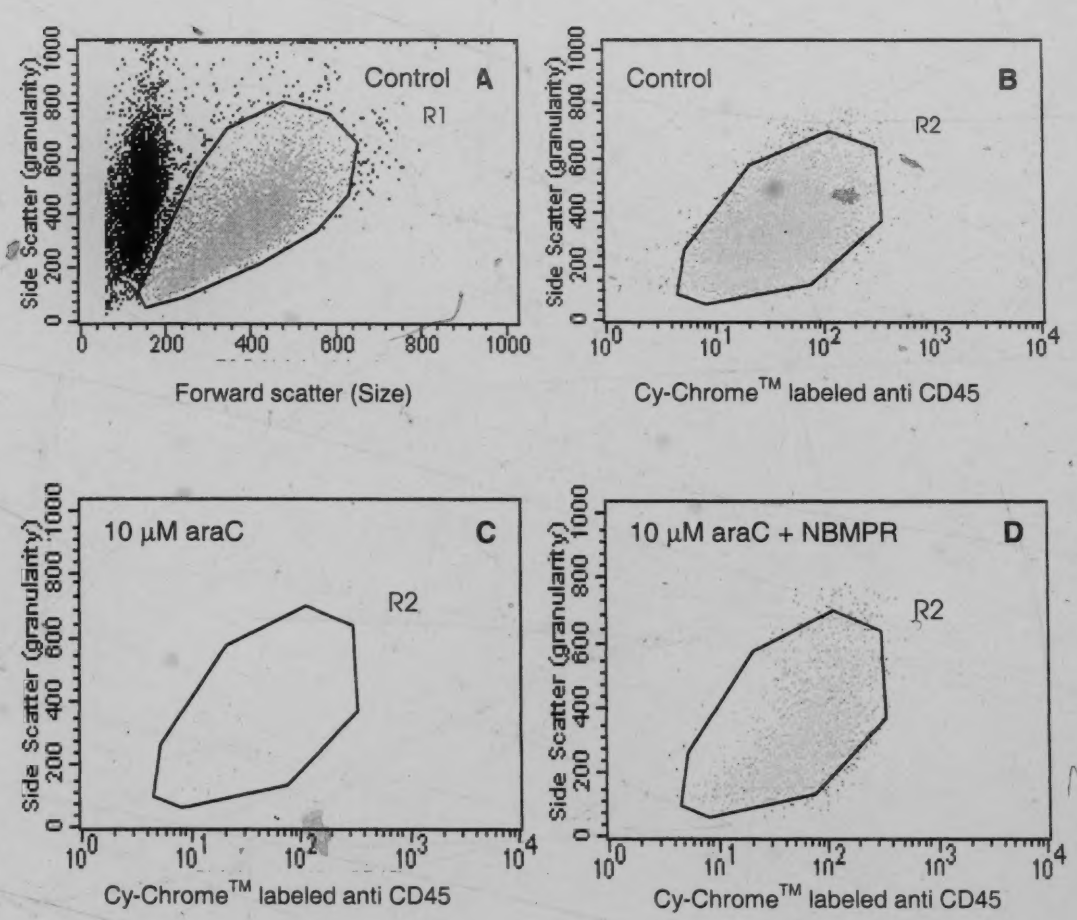



**Figure 11: Flow cytometric identification of leukemic blasts from a patient with AML.** Gating is based on the level of CD45 expression in leukemic cells from sample No. 59. Cells were incubated with graded concentrations of araC ± NBMPR for 96 hr. After the incubation period, cells were stained with Cy-Chrome™ labeled anti CD45 (gate R2) as shown. Panel A and B represent the light scatter plot and the Cy-Chrome™ CD45 staining respectively, of the control. Panel C and D represent the Cy-Chrome™ CD45 staining of araC and araC + NBMPR treated leukemic cells respectively.

**Table 2: Cytotoxicity of araC in leukemic blasts from patients with AML and the effect of 1 μM NBMPR.** $IC_{50}$ values were determined from the concentration-effect plots following 96 hours incubation. A flow cytometry assay was employed to determine the cell viability. Data represented are mean (95 % confidence interval) of duplicate determinations in each experiment.

| Patient No. | Diagnosis | $IC_{50}$, araC (μM) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 1 | AML | 2.1 (0.89-5.2) | > 10* |
| 4 | AML/M5 | 2.2 (0.67-7.1) | > 10* |
| 27 | AML | 2.7 (2.0-3.6) | > 10* |
| 30 | AML/M4 | 0.28 (0.25-0.33) | 1.2 (0.75-2.0)* |
| 31 | AML | 0.32 (0.2-0.38) | 1.5 (0.76-2.8)* |
| 35 | AML | 0.70 (0.50-1.0) | > 10* |
| 36 | AML | 2.6 (1.4-5.0) | > 10* |
| 37 | AML/M3 | 3.4 (2.2-5.2) | > 10* |
| 38 | MDS transformed to AML | 1.9 (1.4-2.5) | > 10* |
| 39 | AML/M1 or M2 | 1.4 (0.83-2.4) | > 10* |
| 40 | AML | > 10 | > 10 |
| 44 | AML | 4.6 (3.3-6.3) | > 10* |
| 45 | AML/M4 | 0.54 (0.37-0.80) | > 10* |
| 46 | AML/M2 | 0.50 (0.36-0.61) | 3.6 (2.1-6.1)* |
| 48 | MDS transformed to AML | 0.05 (0.042-0.052) | 0.12 (0.091-0.16) |
| 49 | AML/M4 | 0.20 (0.13-0.21) | 1.0 (0.75-1.3)* |
| 50 | AML | 0.21 (0.052-0.81) | > 10* |
| 55 | AML | 1.5 (1.0-2.1) | 3.8 (2.0-7.3)* |
| 58 | AML/M5 | 0.10 (0.11-0.14) | 3.5 (1.8 -6.7)* |
| 59 | AML/M4 | 0.10 (0.10-0.12) | 0.54 (0.32-0.92)* |

* NBMPR had statistically significant effect ($p < 0.05$).

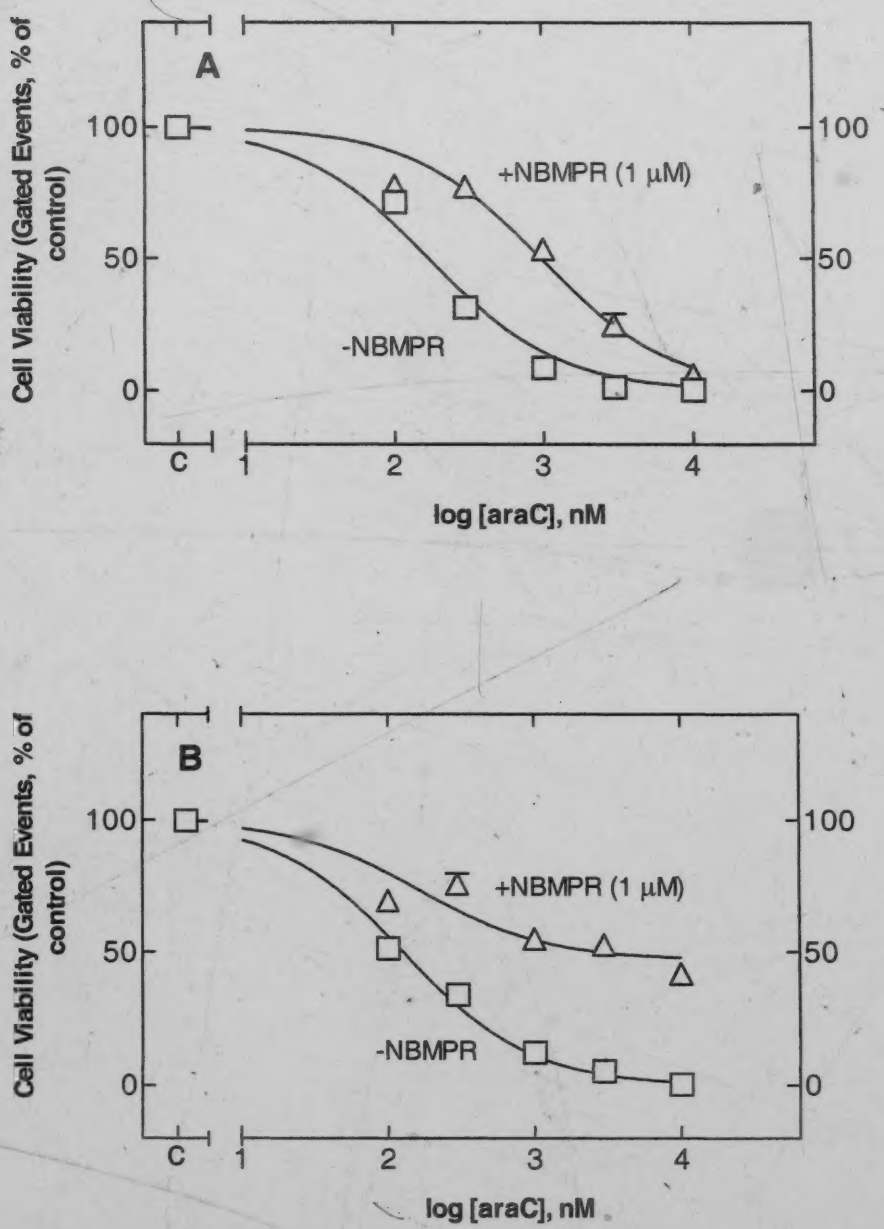



**Figure 12: Sensitivity to araC and the protection from araC cytotoxicity by 1 μM NBMPR in leukemic blasts from patients with AML.** Patient sample no. 49 (Panel A) and Patient sample no. 58 (Panel B) are shown. Cells were incubated for 96 hours in culture medium containing graded concentrations of araC, without (□) and with NBMPR (Δ). After the incubation period, cells were stained with Cy-Chrome™ labeled anti CD45 to identify the leukemic blasts and analyzed by flow cytometry. The data are means ± SD of duplicate measurements at each araC ± NBMPR concentration.

activity was impaired, and araC cytotoxicity was reduced in most samples of fresh leukemic blasts from patients.

*In MDS samples.* Low-density cell populations were isolated from peripheral blood samples from a small number of patients with MDS, and evaluated for araC cytotoxicity (Table 3). In 4 of 6 MDS patient samples, the $IC_{50}$ values for araC were > 10 μM, both in the absence and presence of NBMPR. The $IC_{50}$ values for araC for patient No. 28 and patient No. 57 were approximately 300- and 176-fold higher respectively, as compared to KG1 cells. In those 2 samples, NBMPR significantly protected the cells from the cytotoxic effects of araC, yielding an $IC_{50}$ value of > 10 μM for both samples.

*In ALL samples.* Only a few ALL samples (n=3) were assayed for araC cytotoxicity (Table 4). In 2 of 3 ALL samples examined, NBMPR significantly protected the cells from the cytotoxic effects of araC, increasing $IC_{50}$ values to > 10 μM (No. 47) and 9.4 μM (No. 53). Interestingly, in 1 of 3 ALL samples examined (No. 3) NBMPR potentiated the cytotoxic effects of araC.

In another sample (no. 41) from a patient diagnosed with "leukemia" (not shown in Table 4), NBMPR significantly protected the cells from the cytotoxic effects of araC (+NBMPR, $IC_{50}$ = 0.10 (0.060-0.11) μM; -NBMPR, $IC_{50}$ = 0.03 (0.015-0.10) μM). In sample no. 56, (not shown) diagnosed with transformed polycythemia, the cytotoxicity of araC was evaluated. The $IC_{50}$ for araC was found to be 5.9 (3.7-9.6) μM and NBMPR (1 μM) did not statistically significantly protect the cells from cytotoxic effects of araC, yielding an $IC_{50}$ value of 6.8 (3.2-14.4) μM.

***3.3.2 Initial rate of uptake of [$^3$H]araC in leukemic blasts from patient samples.*** Inward fluxes of [$^3$H]araC were measured in fresh leukemic blasts from patients by

**Table 3: Cytotoxicity of araC in cells from patients with MDS and the effect of 1 μM NBMPR.** $IC_{50}$ values were determined from the concentration-effect plots following 96 hours incubation. The data represent mean (95 % confidence interval) of duplicate determinations in each experiment.

| Patient No. | Diagnosis | $IC_{50}$, araC (μM) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 26 | MDS | > 10 | > 10 |
| 28 | MDS/RARS | 2.2 (1.9-2.7) | > 10* |
| 29 | MDS/RA | > 10 | > 10 |
| 51 | MDS | > 10 | > 10 |
| 52 | MDS/RAEBt | > 10 | > 10 |
| 57 | MDS | 1.2 (0.60-2.6) | > 10* |

* Statistically significant ($p < 0.05$)

**Table 4: Cytotoxicity of araC in leukemic blasts from patients with ALL and the effect of 1 μM NBMPR.** $IC_{50}$ values were determined from the concentration-effect plots following 96 hours incubation. The data represent mean (95 % confidence interval) of duplicate determinations in each experiment.

| Patient No. | Diagnosis | $IC_{50}$, araC (μM) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 3 | ALL | > 10 | 0.92 (0.44 -1.9)* |
| 47 | ALL | 2.4 (1.6-3.7) | > 10* |
| 53 | ALL | 1.1 (0.80-1.5) | 9.4 (6.2-14.2)* |

* Statistically significant ($p < 0.05$).

determining the initial rate of [$^3$H]araC uptake, using the oil stop centrifugation method with time courses of 5-300 sec. Fluxes that were attributable to the *es* nucleoside transport process were recognized in these cells by the use of the *es* nucleoside transport inhibitor, NBMPR (1 μM). As previously described, all the nucleoside influx experiments were performed at 22°C.

*In AML samples.* Inward fluxes of [$^3$H]araC (0.8 μM) were measured in leukemic blasts from 13 AML patients. Table 5 summarizes [$^3$H]araC influx in 13 AML patients, both in the presence and absence of NBMPR. [$^3$H]araC influx was significantly inhibited in 11 of 13 AML patients in the presence of 1 μM NBMPR, indicating that part of the inward flux of araC was mediated by the *es* nucleoside transporter. However, in 2 (No.1 and 38) of 13 AML patients, NBMPR did not significantly affect the flux of 0.8 μM araC. Figure 13 shows examples of time courses of uptake of [$^3$H]araC in the leukemic blasts from patients.

*In MDS samples.* [$^3$H]araC influx was measured in freshly isolated cells from 3 patients with MDS. Table 6 summarizes the flux rates of [$^3$H]araC measured in leukemic blasts from patients with MDS. NBMPR at 1 μM concentration inhibited the flux of [$^3$H]araC in leukemic blasts of patient sample nos. 51 and 57 ($p < 0.05$). However, in 1 of 3 MDS samples (No. 52) NBMPR did not produce any significant effect at the concentration tested (1 μM).

*In ALL samples.* AraC influx was also studied in 2 samples from patients with ALL. In 1 of the 2 ALL samples (No.53), NBMPR significantly inhibited the flux of 0.8 μM araC ($p < 0.05$). In another patient (No. 3) NBMPR did not produce any significant effect on [$^3$H]araC influx at the concentration employed (1 μM). Patient sample no. 41, diagnosed

**Table 5: Inward fluxes of [$^3$H]araC (0.8 μM) in leukemic blasts from patients with AML and the effect of 1 μM NBMPR.** AraC fluxes were measured at 22°C in the absence and presence of 1 μM NBMPR. Cells were pelleted by oil centrifugation and counted for radioactivity. Data represent mean ± SD of triplicate determinations in each experiment.

| Patient No. | Diagnosis | AraC inward flux (fmol [$^3$H]araC/$10^6$ cells/sec) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 1 | AML | 0.10 ± 0.20 | 0.21 ± 0.20 |
| 2 | AML/M2 | 1.0 ± 0.15 | 0.30 ± 0.10* |
| 35 | AML | 0.35 ± 0.030 | 0.12 ± 0.030* |
| 36 | AML | 1.45 ± 0.10 | 0.040 ± 0.11* |
| 38 | MDS transformed to AML | -0.45 ± 0.55 | -0.32 ± 0.31 |
| 39 | AML (M1 or M2) | 0.82 ± 0.10 | -0.030 ± 0.0053* |
| 40 | AML | 0.73 ± 0.11 | 0.10 ± 0.10* |
| 45 | AML/M4 | 8.2 ± 1.9 | 0.40 ± 0.11* |
| 46 | AML/M2 | 2.4 ± 0.10 | 0.22 ± 0.10* |
| 48 | MDS transformed to AML | 2.6 ± 0.042 | 0.30 ± 0.040* |
| 49 | AML/M4 | 0.81 ± 0.10 | 0.30 ± 0.11* |
| 58 | Relapsed AML/M5 | 2.7 ± 0.20 | 0.024 ± 0.071* |
| 59 | AML/M4 | 0.40 ± 0.10 | 0.051 ± 0.031* |

* NBMPR had statistically significant effect on the flux of [$^3$H]araC ( $p < 0.05$).

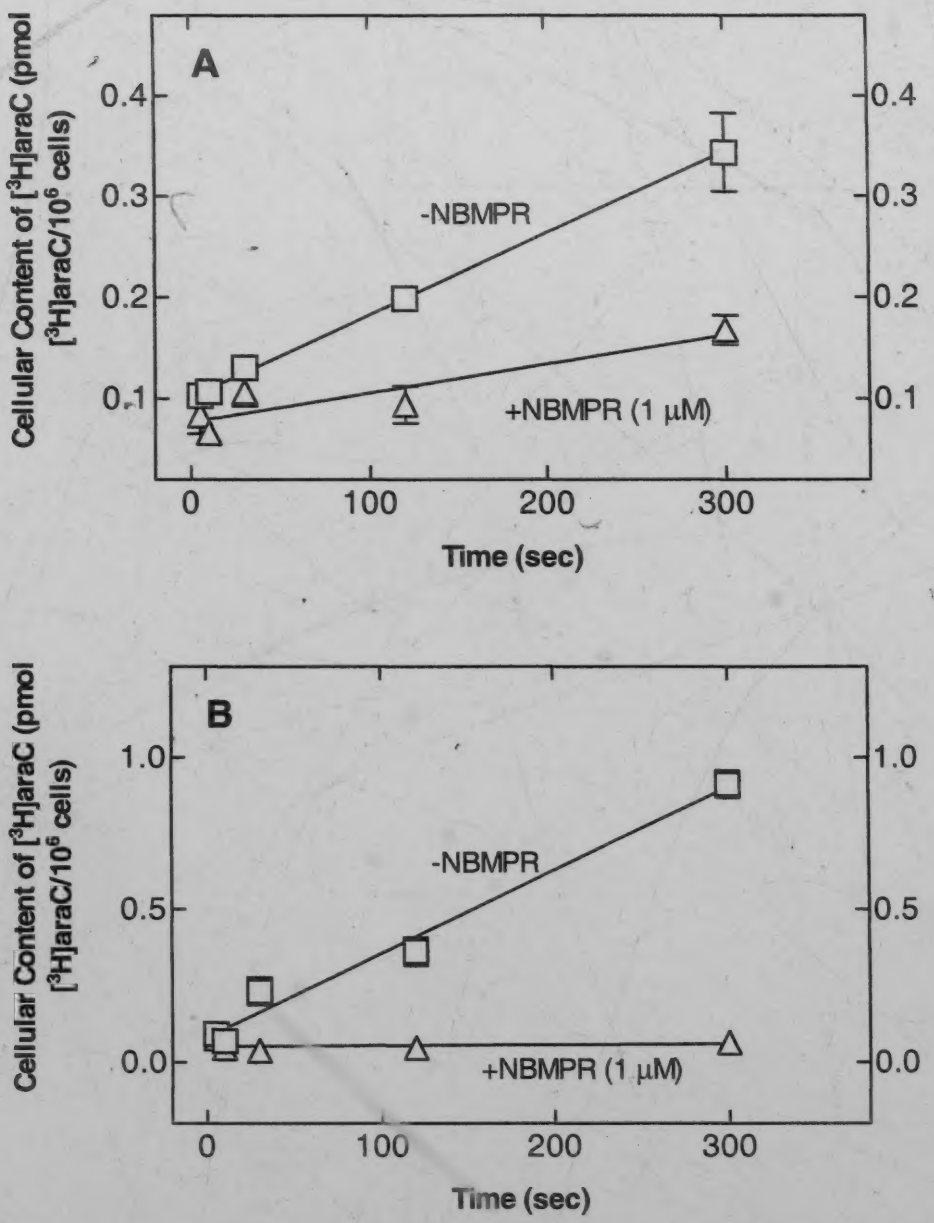



**Figure 13: [$^3$H]AraC uptake in leukemic blasts from patients with AML.** [$^3$H]AraC fluxes were measured in leukemic blasts from patient sample no. 49 (Panel A) and patient sample no. 58 (Panel B) in the absence (□) and presence (Δ) of 1 μM NBMPR. Uptake of 0.8 μM araC was measured at 22°C at the indicated time intervals. Data represent means ± SD of triplicate determinations in each experiment.

**Table 6: Inward fluxes of [$^3$H]araC (0.8 μM) in cells from MDS patients and the effect of 1 μM NBMPR.** AraC fluxes were measured at 22°C. Data represent mean ± SD of triplicate determinations in each experiment.

| Patient No. | Diagnosis | AraC inward flux (fmol [$^3$H]araC/$10^6$ cells/sec) | |
|---|---|---|---|
| | | - NBMPR | +NBMPR |
| 51 | MDS | 0.45 ± 0.045 | 0.070 ± 0.060* |
| 52 | MDS/RAEBt | 0.32 ± 0.055 | 0.28 ± 0.11 |
| 57 | MDS | 0.90 ± 0.10 | 0.50 ± 0.10* |

* Effect of NBMPR was statistically significant ($p < 0.05$).

**Table7: Inward fluxes of [$^3$H]araC (0.8 μM) in leukemic blasts from patients with ALL and the effect of 1 μM NBMPR.** AraC fluxes were measured at 22°C. Data represent mean ± SD of triplicate determinations in each experiment.

| Patient No. | Diagnosis | AraC inward flux (fmol [$^3$H]araC/$10^6$ cells/sec) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 3 | ALL | 0.43 ± 0.40 | 0.30 ± 0.10 |
| 53 | ALL | 1.3 ± 0.013 | 0.40 ± 0.090* |
| 41 | Leukemia | 0.41 ± 0.11 | 0.20 ± 0.043* |

* Effect of NBMPR was statistically significant ($p < 0.05$).

with "leukemia" is also shown in Table 7 and NBMPR significantly inhibited the flux of [$^3$H]araC.

***3.3.3 Correlation of araC influx and cytotoxicity in fresh leukemic blasts from patients.***

An aim of this study was to investigate whether membrane transport of araC is a determinant of cytotoxicity of araC in leukemic blasts from patients. Specifically, an attempt has been made to show that there exists a correlation between *es* transporter-mediated component of araC uptake into cells and araC cytotoxicity that could be blocked by the use of an *es* nucleoside transport inhibitor. Transport and cytotoxicity parameters for 15 patients (10 samples with AML, 3 samples with MDS, 1 sample with ALL and 1 with "leukemia") were studied. Time courses of [$^3$H]araC uptake were measured in the absence or presence of NBMPR as described above, to determine initial rates (inward fluxes) of *es* transporter-mediated and non *es*-mediated araC uptake in leukemic blasts from patients. The *es* transporter-mediated uptake of araC was measured as the difference between the total flux of araC and the flux inhibited by 1 μM NBMPR. The *es* transporter-mediated (NBMPR-sensitive) component of araC (0.8 $\mu$M) uptake was plotted against the percentage of araC (1 $\mu$M) cytotoxicity that could be prevented by NBMPR in the measurements of araC cytotoxicity (concentration-effect curves) described above. Figure 14 shows that the NBMPR-sensitive component of araC cytotoxicity was correlated with the *es* transporter-mediated component of araC uptake in leukemic blasts from 15 patients ($r = 0.51$, $p < 0.05$), providing strong support for the hypothesis that *es* transporter-mediated influx is a determinant of sensitivity to araC in leukemic blasts from patients.

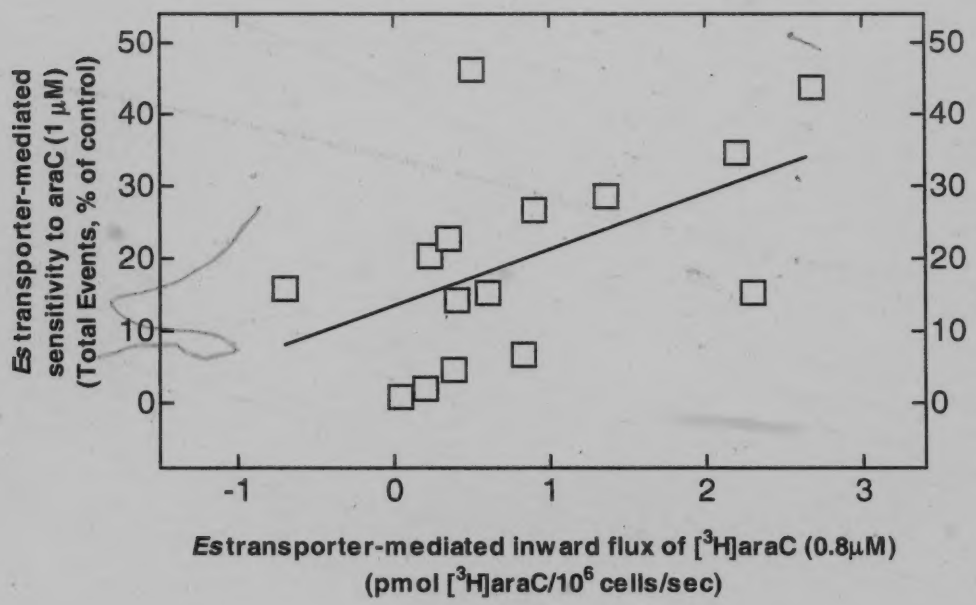



**Figure 14: Correlation of *es* nucleoside transporter-mediated inward flux of araC and the NBMPR-sensitive component of araC (1 μM) cytotoxicity in leukemic blasts from patients.** A statistically significant correlation ($p < 0.05$) between the cytotoxicity and inward flux of araC was found in the leukemic blasts from patient samples ($r = 0.51$, $n = 15$).

**3.4 Cytotoxicity and inward fluxes of dFdC in fresh leukemic blasts from patients**

***3.4.1 DFdC cytotoxicity in fresh leukemic blasts from patients.*** The goal of this set of experiments was to examine whether nucleoside transport was a determinant of the cytotoxicity of dFdC in leukemic cells from patients. The cytotoxicity experiments were performed using flow cytometry assays as described for the experiments with araC. Cells were incubated for 96 hr in the presence of graded concentrations of dFdC and dFdC cytotoxicity was manipulated by exposure of the leukemic blasts to nucleoside transport inhibitors such as NBMPR. The surviving cells were stained with Cy-Chrome™ labeled anti-CD45, which enabled the identification and enumeration of leukemic blasts by flow cytometry (Figure 15).

*In AML samples.* In 4 of 8 AML patient samples studied, the $IC_{50}$ values for dFdC cytotoxicity were > 10 μM both in the presence and absence of transport inhibitor, NBMPR (Table 8). In one sample (No. 9), the cytotoxic effects of dFdC were examined in the presence of three transport inhibitors: NBMPR, DPM, and dilazep at all concentrations of 1 μM. The $IC_{50}$ values both in the presence and the absence of transport inhibitors were > 10 μM. Therefore, NBMPR or the other inhibitors did not significantly affect dFdC cytotoxicity in 6 of 8 AML samples tested. However, in 2 of the 8 patient samples NBMPR statistically significantly protected the cells from dFdC cytotoxicity ($p < 0.05$). Figure 16 (Panel A and Panel B) demonstrates the cytotoxic effects of dFdC and protection by NBMPR.

*In ALL samples.* In 1 of 2 ALL samples (No.20), the leukemic cells were sensitive to the cytotoxic effects of dFdC and NBMPR protected the cells from the cytotoxic effects of dFdC with the $IC_{50}$ value > 10 μM (Table 9). Interestingly, in 1 of 2 ALL patient

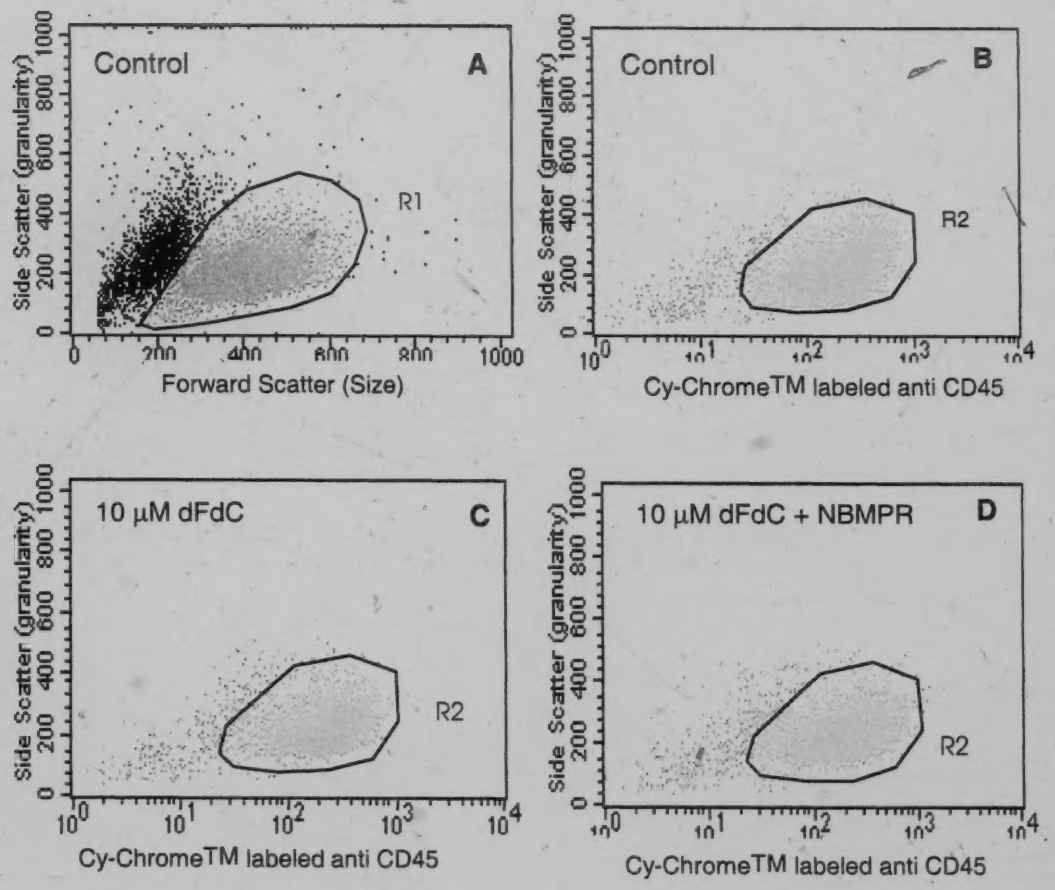



**Figure 15: Flow cytometric identification of leukemic blasts from a patient with AML.** Gating is based on the level of CD45 expression in leukemic cells from sample No.8. Cells were incubated with graded concentrations of dFdC ± NBMPR for 96 hr. After the incubation period, cells were stained with Cy-Chrome™ labeled anti CD45 (gate R2) as shown. Panel A and B represent the light scatter plot and the Cy-Chrome™ CD45 staining respectively, of the control. Panel C and D represent the Cy-Chrome™ CD45 staining of dFdC and dFdC + NBMPR (1 μM) treated leukemic cells respectively.

**Table 8: Cytotoxicity of dFdC in leukemic blasts from patient samples with AML and the effect of 1 μM NBMPR.** In patient sample No. 9, the dFdC cytotoxicity assay was performed both in the absence and presence of the *es* nucleoside transport inhibitors, NBMPR, DPM and dilazep. The data are mean (95 % confidence interval) of duplicate measurements at each dFdC ± NBMPR concentration.

| Patient No. | Diagnosis | $IC_{50}$, dFdC (μM) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 5 | AML/M5 | > 10 | > 10 |
| 6 | Relapsed AML | 0.015 (0.010-0.022) | 0.025 (0.011-0.055) |
| 8 | AML | > 10 | > 10 |
| 9 | AML/M2 | > 10 | > 10 |
| 10 | MDS or AML | 2.8 (0.43-9.0) | 9.3 (2.8-10.4) |
| 12 | AML | > 10 | > 10 |
| 13 | AML | 4.4 (1.3 -9.4) | > 10* |
| 14 | AML/M4 | 1.6 (0.75- 3.5) | > 10* |

* Effect of NBMPR was statistically significant ($p < 0.05$).

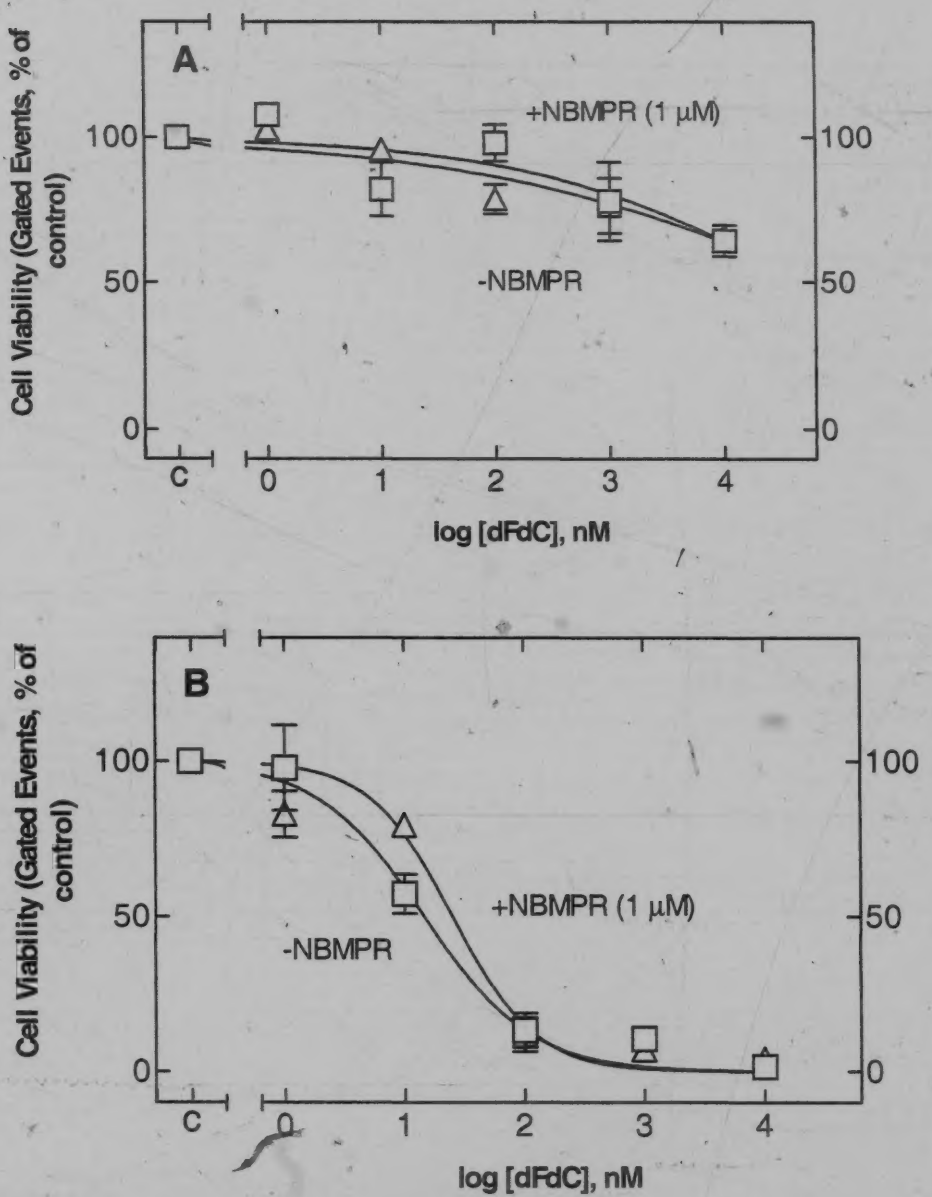



**Figure 16: Sensitivity to dFdC and protection from dFdC cytotoxicity by 1 μM NBMPR in leukemic blasts from patients with AML.** Leukemic blasts from patient sample no. 12 (Panel A) and patient sample no. 6 (Panel B) were incubated for 96 hours in culture medium containing graded concentrations of dFdC, without (□) and with NBMPR (Δ). After the incubation period, cells were stained with Cy-Chrome™ labeled anti CD45 to identify the leukemic blasts. The data are means ± SD of duplicate measurements at each dFdC ± NBMPR concentration.

**Table 9: Cytotoxicity of dFdC in leukemic blasts isolated from patient samples and the effect of 1μM NBMPR.** $IC_{50}$ values were determined from the concentration-effect plots. Microcultures were incubated for 96 hours and analyzed by flow cytometry. The data are mean (95 % confidence interval) of duplicate measurements at each dFdC ± NBMPR concentration.

| Patient No. | Diagnosis | $IC_{50}$, dFdC (μM) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 7 | ALL | > 10 | 1.9 (0.60-6.1) |
| 20 | ALL | 7.4 (4.0 –10.6) | > 10* |
| 11 | Lymphoma | > 10 | > 10 |

*Effect of NBMPR was statistically significant ($p < 0.05$).

samples (No.7), NBMPR potentiated the cytotoxic effects of dFdC with an $IC_{50}$ value of 1.9 μM. However, this potentiation of dFdC cytotoxicity was not statistically significant. In one of the patient samples (No.11), diagnosed with lymphoma, NBMPR had no significant effect on dFdC cytotoxicity.

***3.4.2 Initial rate of uptake of [$^3$H]dFdC in leukemic blasts from patient samples.*** The initial rate of [$^3$H]dFdC uptake (inward flux of dFdC) was determined in leukemic blasts from patient samples and these rates were compared with the fluxes of dFdC that were measured in KG1 cells (Section 3.2.2). These influx rates were 4 - 28 fold lower than the flux rates observed in KG1 cells (Table 10 and 11). NBMPR (1 $\mu$M) was employed to identify the *es* nucleoside transporter-mediated component of dFdC uptake in the blast samples. NBMPR significantly inhibited the uptake of [$^3$H]dFdC (Table 11) in 2 of 3 AML samples (No. 6 and No. 15). These results indicate that *es* nucleoside transporter activity significantly contributed to dFdC uptake in these cells (Figure 17). The flux rates in the presence and absence of NBMPR (1 μM) were statistically significantly different in sample nos. 6 and 15 ($p < 0.05$). NBMPR did not significantly affect the flux rates of [$^3$H]dFdC in 2 of 4 samples ( sample no. 7 and 8) studied.

***3.4.3 Comparison of dFdC cytotoxicity and es transporter-mediated inward flux in leukemic blasts from patients.*** Both dFdC cytotoxicity and inward fluxes of dFdC were studied in samples from 3 patients as summarized in Table 12. A correlation between dFdC cytotoxicity and inward fluxes of dFdC was not attempted because of the small number of samples tested, the lack of sensitivity to dFdC of some samples, and the inconsistent effects of NBMPR.

**Table 10: Inward fluxes of [$^3$H]dFdC (0.8 μM) in leukemic blasts from patients.** DFdC fluxes were measured at 22°C. Data represent mean ± SD of triplicate determinations in each experiment.

| Patient No. | Diagnosis | DFdC inward flux (fmol [$^3$H]dFdC/$10^6$ cells/sec) |
|---|---|---|
| 16 | Early MDS | 0.28 ± 0.21 |
| 17 | MDS/RAEB | 1.3 ± 1.2 |
| 18 | AML/M5b | 1.3 ± 0.70 |
| 19 | Leukemia | 0.33 ± 0.23 |

**Table 11: Inward fluxes of [$^3$H]dFdC (0.8 μM) in leukemic blasts from patients and the effect of 1 μM NBMPR.** dFdC fluxes were measured at 22°C in the absence and presence of 1 μM NBMPR. Data represent mean ± SD of triplicate determinations in each experiment.

| Patient No. | Diagnosis | DFdC inward flux (fmol [$^3$H]dFdC/$10^6$ cells/sec) | |
|---|---|---|---|
| | | -NBMPR | +NBMPR |
| 6 | Relapsed AML | 0.51 ± 0.072 | 0.16 ± 0.076* |
| 8 | AML | 0.31 ± 0.10 | 0.24 ± 0.030 |
| 15 | AML/M4 | 0.28 ± 0.014 | 0.50 ± 0.040* |
| 7 | ALL | 0.19 ± 0.060 | -0.011 ± 0.19 |

*Effect of NBMPR was statistically significant ($p < 0.05$).

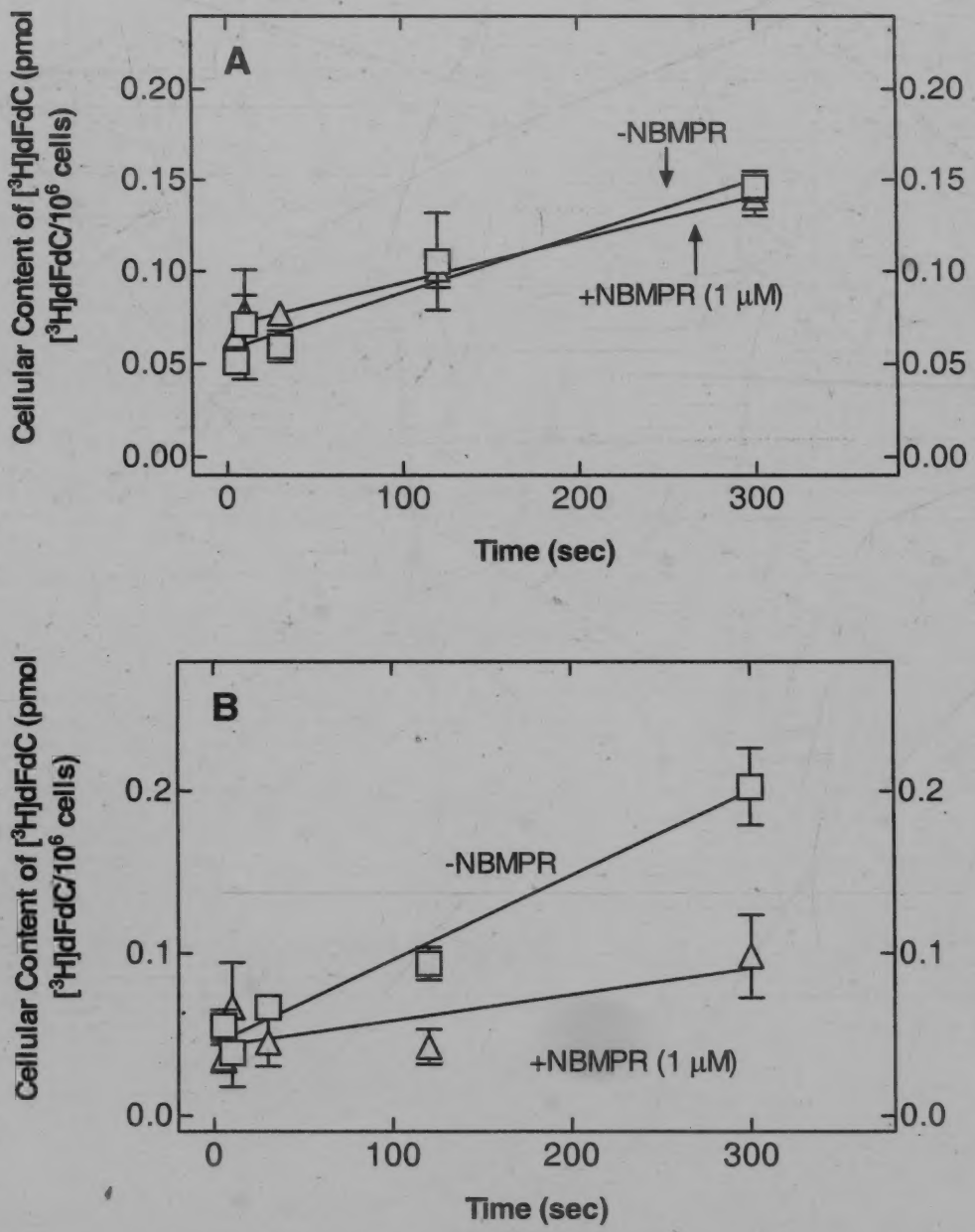



**Figure 17: Uptake of [$^3$H]dFdC in leukemic blasts from patients with AML.** Shown are Patient sample no.8 (Panel A) and Patient sample no. 6 (Panel B). Assay mixtures contained 2 x $10^6$ cells in RPMI$^t$ medium, without inhibitor, NBMPR (□) or with 1 μM NBMPR (Δ). Uptake of 0.8 μM dFdC was measured at 22°C at the indicated time intervals. Data represent means ± SD of triplicate determinations in each experiment.

**Table 12: Comparison of inward fluxes of dFdC and cytotoxicity in leukemic blasts from patients.**

| Patient No. | Diagnosis | $IC_{50}$, dFdC (μM) | | DFdC inward flux (fmol [$^3$H]dFdC/$10^6$ cells/sec) | |
|---|---|---|---|---|---|
| | | -NBMPR | +NBMPR | -NBMPR | +NBMPR |
| 6 | Relapsed AML | 0.015 (0.010-0.022) | 0.025 (0.011 -0.055) | 0.51 ± 0.072 | 0.16 ± 0.076* |
| 7 | ALL | > 10 | 1.9 (0.60-6.1) | 0.19 ± 0.060 | -0.11 ± 0.19 |
| 8 | AML | > 10 | > 10 | 0.31 ± 0.10 | 0.24 ± 0.030 |

* Effect of NBMPR was statistically significant ($p < 0.05$).

**3.5 Selection and characterization of an *es* nucleoside transporter-deficient, dFdC-resistant cell line (CEM/CCL1/G cells)**

***3.5.1 Selection of dFdC-resistant cells from CEM/CCL1 cells.*** The CEM/CCL1 subline was isolated previously in this laboratory by using fluorescence activated cell sorting (flow cytometry) to select cells from the human leukemic T-lymphoblast cell line, CCRF-CEM that were dimly stained with SAENTA-fluorescein (5′-S-(2-aminoethyl)-$N^6$- (4-nitrobenzyl)-5′-thioadenosine)-fluorescein), a tightly-bound ligand of the *es* nucleoside transporter. At the time of their isolation, CEM/CCL1 cells were partly characterized and found to be partly *es* deficient. As a part of this thesis project, CEM/CCL1 cells were cultured in the presence of stepwise increasing concentrations of dFdC to select a dFdC-resistant subpopulation. Cells were initially exposed to 10 nM dFdC, and after a stable growth rate was achieved, the concentration of dFdC was increased in a step wise manner i.e. 10 nM, 20 nM, 50 nM, 100 nM, 200 nM and 500 nM. Figure 18 represents the selection process in duplicate CEM/CCL1 cultures, which proceeded over a period of about two months, yielding the dFdC-resistant subline, CEM/CCL1/G. As will be shown, characteristics of the duplicate, dFdC-resistant cultures did not differ significantly, and so they will be referred as a single subline. The MGT of CCRF/CEM cells was 23 ± 0.82 (mean ± SD). The CEM/CCL1 cells had a MGT of 25 ± 0.54, which was not statistically different from CCRF/CEM cells. However, the MGT of the CCL1/CEM/G cells and dFdC withdrawn, CEM/CCL1/Gw cells (discussed later) were 27 ± 2.3 and 29 ± 2.9 respectively, which were statistically significantly different than the CCRF/CEM cells.

***3.5.2 SAENTA-fluorescein binding studies in dFdC-resistant cells.*** The cellular content

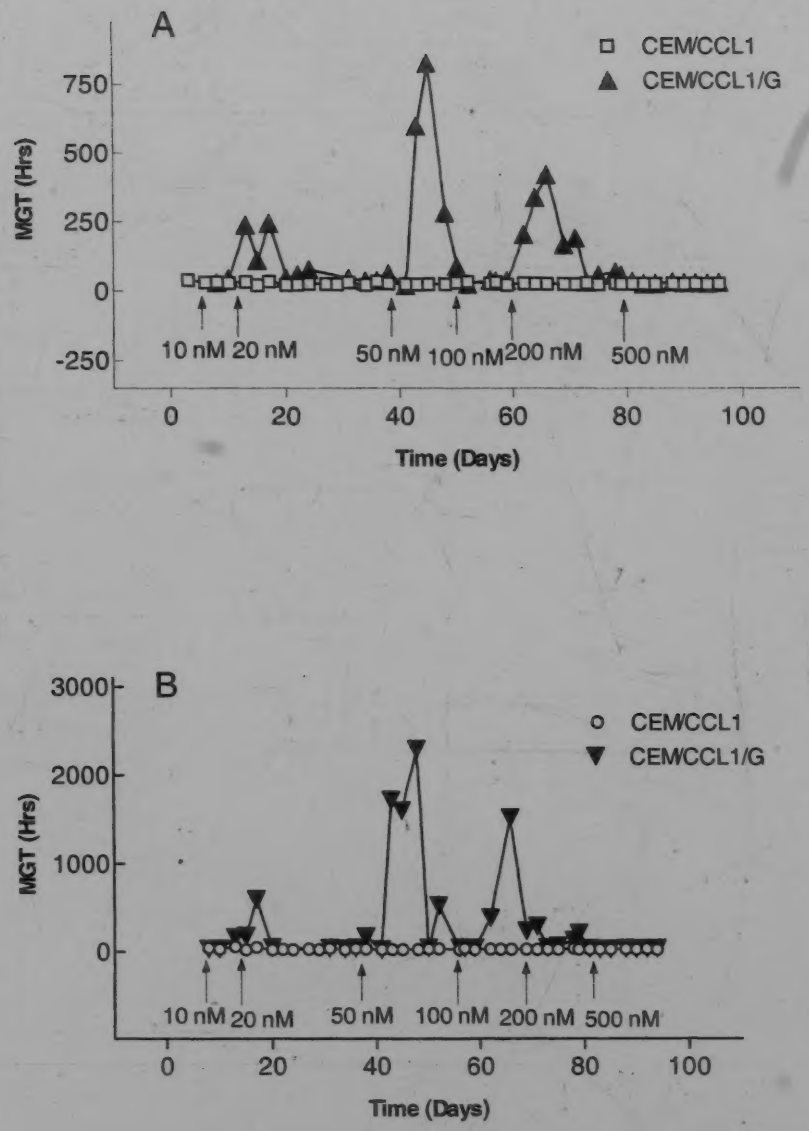



**Figure 18: Selection of CEM/CCL1/G cells from the CEM/CCL1 subline.** The cells were selected in two separate incubators: 9-21 A (Panel A) and 9-21 C (Panel B), by culturing in the presence of dFdC. Drug pressure was applied by increasing the concentration of dFdC in a stepwise manner, as shown by the arrows.

of *es* nucleoside transporters was determined in CEM/CCL1/G cells in an equilibrium binding assay that used SAENTA-fluorescein in a flow cytometry procedure. In that procedure, the RFI value obtained from each fluorescence histogram is a quantitative measure of bound SAENTA-fluorescein. Specific binding was calculated as the difference between the total binding (in the absence of NBMPR) and the non-specific binding (in the presence of NBMPR). The specific binding (*es* nucleoside transporter abundance) of CEM/CCL1/G cells was compared with that of CEM/CCL1 cells, from where they were selected. The specific binding of the CEM/CCL1/G cells was 33 ± 19 mean RFI units/cell (mean ± SD), which was significantly less than the mean RFI of the control group, CEM/CCL1 cells (230 ± 75, RF1 units/cell), n = 4 (Figure 19). Thus, these results indicate that the dFdC selected cells were *es* nucleoside transporter-deficient as compared to the CEM/CCL1 cells and CCRF/CEM cells. In order to examine the stability of *es* nucleoside transporter deficiency in the selected cell line, dFdC was withdrawn from the culture medium and the cells (CEM/CCL1/Gw) were assayed for *es* nucleoside transporter abundance for approximately 6 weeks, as shown in Figure 20. During this period, the *es* transporter abundance was measured and compared in 3 cell lines-CEM/CCL1 cells, CEM/CCL1/G cells and CEM/CCL1/Gw cells. It was observed that there was no statistically significant difference between the *es* nucleoside transporter abundance between CEM/CCL1/G cells and CEM/CCL1/Gw cells indicating that the *es* nucleoside transporter deficiency was stable in the CEM/CCL1/G cells in the absence of dFdC (Figure 19).

***3.5.3 Cell cycle phase distribution studies.*** Cell cycle phase distribution studies of the dFdC-selected cells were conducted after the determination of *es* transporter abundance

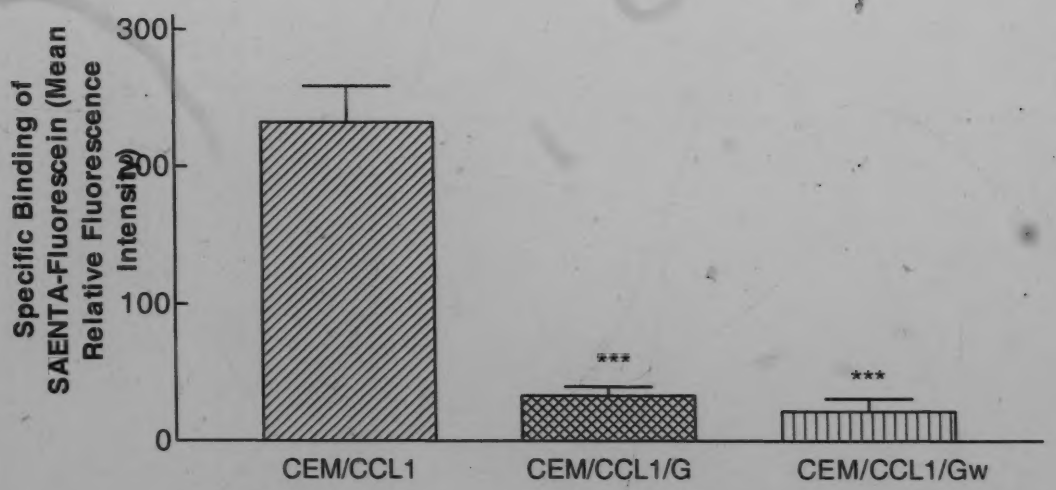



**Figure 19: *Es* nucleoside transporter abundance in CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells.** Transporter abundance was measured by flow cytometry in an equilibrium-binding assay that employed SAENTA-fluorescein. The data represent mean ± SD (n = 8). Data were pooled from four experiments, each performed in duplicate. The *es* transporter abundance was statistically significantly lower in CEM/CCL1/G cells and CEM/CCL1/Gw cells as compared to CEM/CCL1 cells. Statistical significance was determined using Tukey-Kramer multiple comparison test with level of significance set at $p < 0.001$.

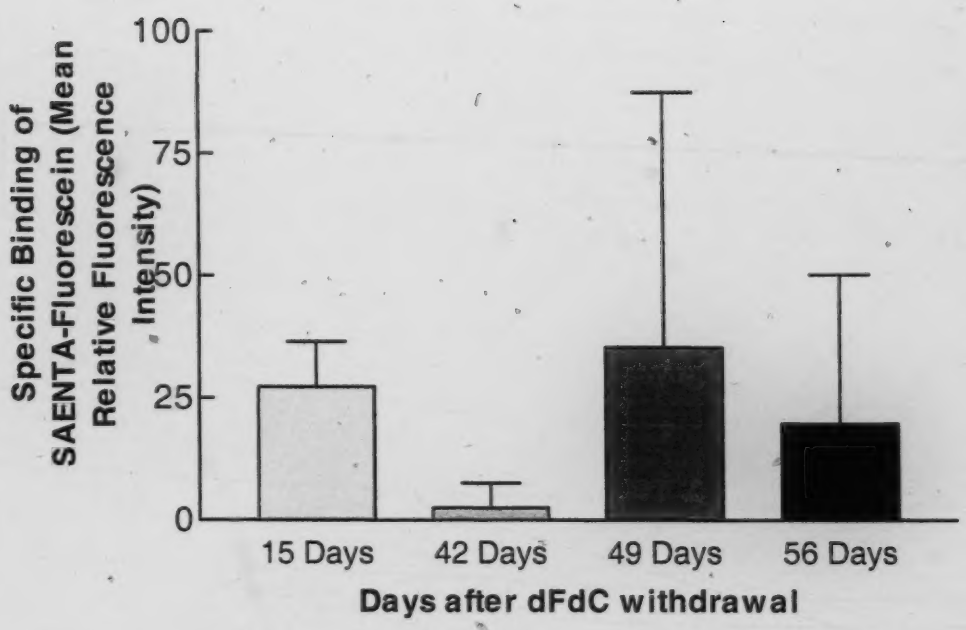



**Figure 20: Stability of *es* nucleoside transporter abundance in CEM/CCL1/Gw cells.** Transporter abundance was measured by flow cytometry in an equilibrium-binding assay that employed SAENTA-fluorescein. The data represent mean ± SD of quadruplicate measurements in each experiment, except for the measurement after 15 days, which was carried out in duplicate.

in CEM/CCL1/G cells, to determine whether the growth properties of the selected cells had changed. CEM/CCL1, CEM/CCL1/G, CEM/CCL1/Gw cells were harvested, washed and fixed in 70% ethanol. The cells were then stained with PI and analyzed by flow cytometry, using Modfit 2.0 software. As shown in Figure 21, the dFdC selected cells, CEM/CCL1/G, did not significantly differ in any phase of the cell cycle $G_0/G_1$, S and $G_2$/M phase as compared to the CEM/CCL1 cells. Also, there was no statistically significant difference in the cell cycle phase distribution between the CEM/CCL1/Gw and CEM/CCL1 cells. The statistical analysis was performed using ANOVA and the cell cycle distributions (of all the phases of cell cycle) of three groups were compared individually using Tukey-Kramer multiple comparison test.

***3.5.4 Cytotoxicity assays.*** Cytotoxicity assays were performed on dFdC selected cells to compare their drug sensitivity with CEM/CCL1 cells. Further, assays were conducted to determine the cross resistance of the dFdC selected cell line (CEM/CCL1/G) to other nucleosides that use the *es* nucleoside transporter for access to cells. The other nucleoside drugs employed were araC, 2-CdA and dThd. 2-CdA and araC were chosen for the assay as they are also cytotoxic antileukemic drugs, and require the *es* nucleoside transporter for entry into the cells as well as dCK for activation. On the other hand dThd does not require dCK for activation but does require the *es* nucleoside transporter for its entry into the cells. As shown in Figure 22, the CEM/CCL1 cells were sensitive to the cytotoxic effects of dFdC and araC, with $IC_{50}$ values 4.1 ± 1.1 nM and 8.1 ± 1.1 nM respectively. However, the CEM/CCL1/G cells were resistant to both dFdC and araC, with $IC_{50}$ values > 100 nM. In addition, as shown in Figure 23, the CEM/CCL1/G cells

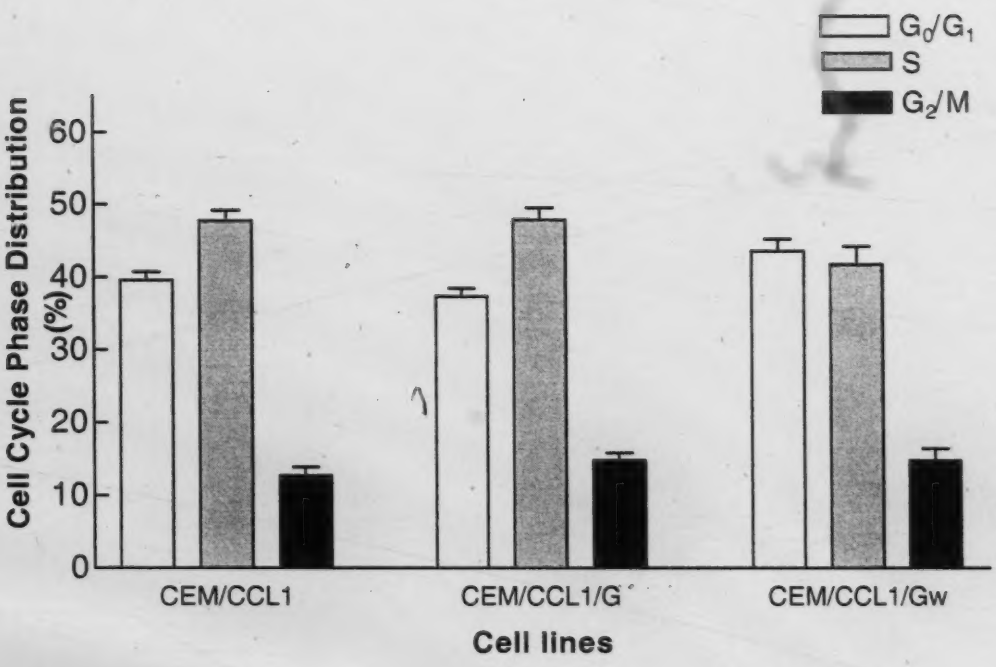



**Figure 21: Cell cycle phase distributions in CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells.** The cells were stained with PI and analyzed by flow cytometry. Modfit 2.0 software was used to determine the cell cycle phase distribution of cells. The data represented are mean ± SD (n = 8). Data were pooled from four experiments, each performed in duplicate. The statistical analysis was performed using Tukey-Kramer test with the level of significance at $p < 0.05$).

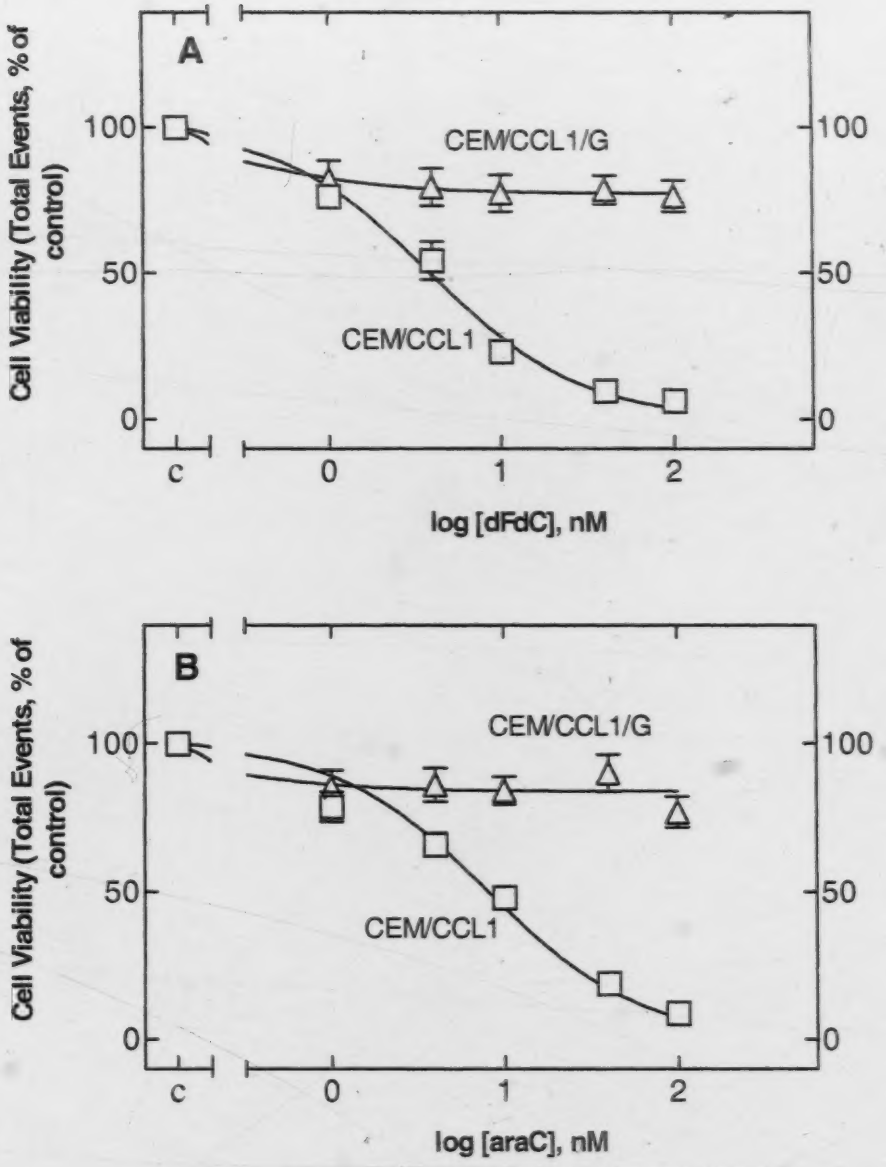



**Figure 22: Cytotoxicity of dFdC and araC in CEM/CCL1 and CEM/CCL1/G cells.** CEM/CCL1 cells (□) and CEM/CCL1/G cells (Δ) were exposed to graded concentrations of dFdC (Panel A) or araC (Panel B) for 72 hours. After the incubation period, cells were washed free of drug, resuspended in HSC buffer and analyzed by flow cytometry. The data represented are the mean ± SD (n = 8). Data were pooled from four experiments, each performed in duplicate.

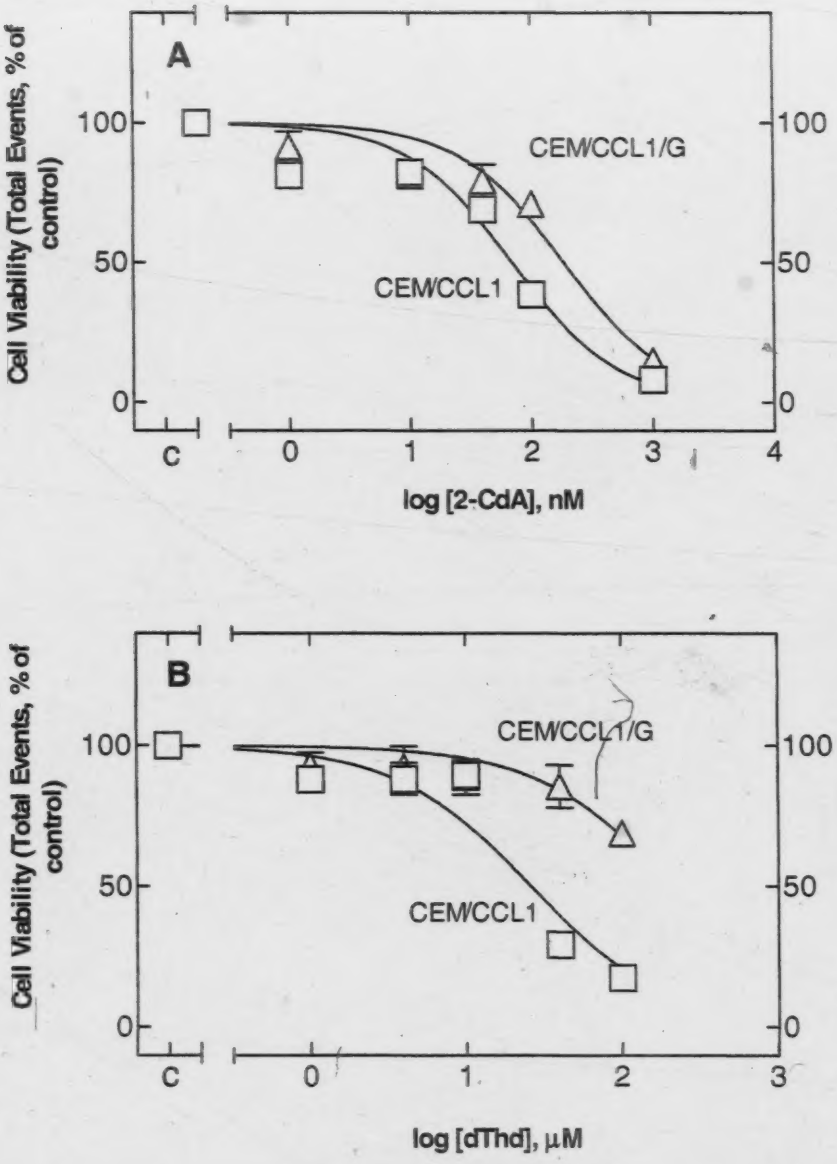



**Figure 23: Cytotoxicity of 2-CdA and dThd in CEM/CCL1 and CEM/CCL1/G cells.** CEM/CCL1 cells (□) and CEM/CCL1/G cells (Δ) were exposed to graded concentrations of 2-CdA (Panel A) or dThd (Panel B) for 72 hours. After the incubation period, cells were washed free of drug, resuspended in HSC buffer and analyzed by flow cytometry. The data represented are mean ± SD (n = 8). Data were pooled from four experiments, each performed in duplicate.

were more than 4- fold resistant to the cytotoxic effects of dThd ($IC_{50}$ > 100 μM), when compared to CEM/CCL1 cells ($IC_{50}$, 26 ± 1.1 μM). CEM/CCL1/G cells were about 3-fold resistant to the cytotoxic effect of 2-CdA ($IC_{50}$ value of 190 ± 1.3 nM), when compared with CEM/CCL1 cells ($IC_{50}$ value of 65 ± 1.2 nM) (Figure 23). As seen in Figure 22 and 23, the cytotoxicity assay curves for dFdC and araC appear biphasic in nature. The magnitude of the sensitive component for dFdC and araC was approximately 23 % and 16 % respectively.

***3.5.5 Deoxycytidine kinase assay.*** A deficiency in dCK activity, which plays a key role in metabolism of some nucleoside analogs inside cells, has been shown to be a mechanism of resistance to araC in experimental systems.[102] Specifically, dCK catalyzes the rate-limiting step in the sequential formation of mono-, di-, and triphosphate derivatives of certain nucleosides including dFdC, araC and 2-CdA. To determine whether resistance of CEM/CCL1/G cells to dFdC, araC and 2-CdA in the present study might be partly attributable to a loss of activity of that enzyme, the levels of dCK were measured in CEM/CCL1/G cells, CEM/CCL1 cells, and CCRF/CEM cells. Rates of formation of 5'-phosphate derivatives of [$^3$H]2'-deoxycytidine were measured in cell extracts in a radiochemical assay, in which the isotopically labeled nucleotides were retained on ion exchange papers and the precursors washed away. Results are expressed in terms of the amount of protein in each cell extract, which was measured by the Bradford assay.[96] Figure 24 shows the dCK activity in the three cell lines. The activities of the enzyme in CCRF/CEM cells and CEM/CCL1 cells were not statistically significantly different ($p < 0.05$). However, in the dFdC selected cell line the activity of the enzyme was significantly reduced to 55 % and 54 % of the values in CCRF/CEM and

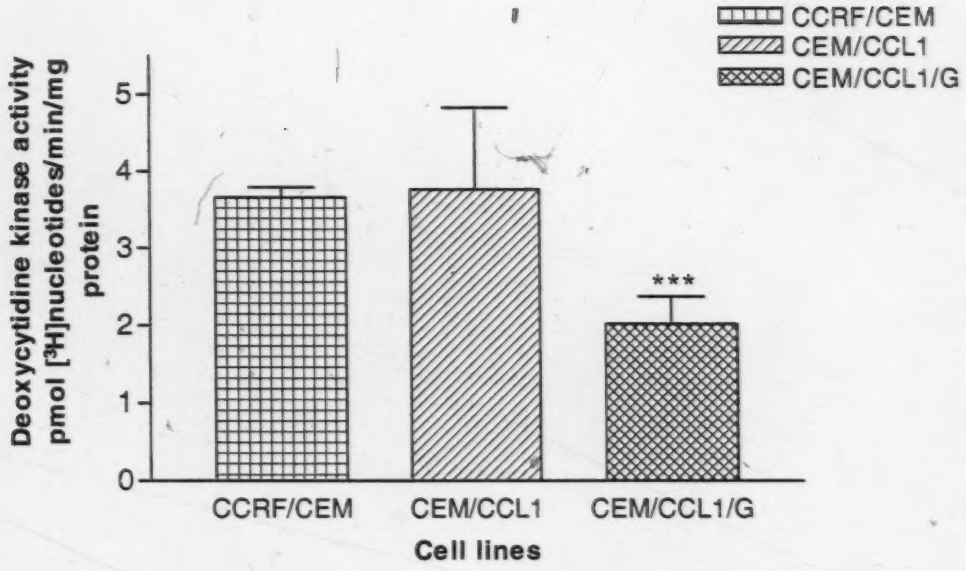



**Figure 24: Deoxycytidine kinase activity in CCRF/CEM, CEM/CCL1 and CEM/CCL1/G cells.** The data represent mean ± SD (n = 16). Data was pooled from four experiments, each performed in quadruplicate. The statistical analysis was performed using Tukey-Kramer test with the level of significance at $p < 0.0001$.

CEM/CCL1 cells respectively. Table 13 summarizes the data for *es* nucleoside transporter abundance, cell cycle phase distribution, cytotoxicity and dCK activity in CCRF/CEM, CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells.

**Table 13: Summary of parameters evaluated in CCRF/CEM, CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells.**

| Parameters | CCRF/CEM | CEM/CCL1 | CEM/CCL1/G | CEM/CCL1/Gw |
|---|---|---|---|---|
| **MGT** (hours), n=10 | 23 ± 0.82* | 25 ± 0.54 | 27 ± 2.3 | 29 ± 2.9 |
| **Specific binding of SAENTA-fluorescein** (Relative fluorescence units), n = 4 | 850 ± 150†† | 230 ± 75 | 33 ± 19 | 22 ± 26 |
| **PI Staining** (% of cells) n = 4-10 $G_0/G_1$ | 37 ± 1†† | 40 ± 3.5 | 37 ± 3.3 | 44 ± 4.0 |
| S | 45 ± 4†† | 48 ± 4.5 | 48 ± 5.2 | 42 ± 6.2 |
| $G_2/M$ | 18 ± 3†† | 13 ± 3.5 | 15 ± 3.2 | 15 ± 4.0 |
| **Cytotoxicity Assay** n = 2-4 | | | | |
| $IC_{50}$, dFdC (nM) | ND† | 4.1 ± 1.1 | > 100 | ND |
| $IC_{50}$, araC (nM) | 3.0 ± 0.1†† | 8.1 ± 1.1 | > 100 | ND |
| $IC_{50}$, 2-CdA (nM) | 18 ± 1†† | 65 ± 1.2 | 190 ± 1.3 | ND |
| $IC_{50}$, dThd (μM) | ND | 26 ± 1.1 | > 100 | ND |
| **Deoxycytidine kinase activity** (pmol [$^3$H]nucleotides/ min/mg protein n = 4 | 3.7 ± 0.21 | 3.8 ± 1.0 | 2.0 ± 0.32 | ND |

* Significantly different as compared to CEM/CCL1/G and CEM/CCL1/Gw cells ($p < 0.05$). In this table, "n" indicates the number of experiments performed.
† Not determined. These cells were not assayed for the respective parameter.
†† Values previously determined in the laboratory.

# Chapter 4

# DISCUSSION

Certain nucleoside analogs are important antileukemic drugs. However, clinical resistance to nucleoside analog therapy remains a major obstacle in the successful treatment of leukemia, and mechanisms of resistance to cytotoxic nucleosides are poorly understood. The general goal of this thesis project was to determine whether the *es* nucleoside transporter-mediated fluxes of araC and dFdC were determinants of the cytotoxicity of those agents in fresh leukemic blasts from patients and in human leukemia cell lines. In that way, this study has tested the hypothesis that a deficiency in transporter-mediated fluxes of nucleosides is a mechanism of resistance to nucleoside drugs. These studies of transporter function complement earlier work in which associations between transporter abundance and cytotoxicity were examined.

*Cytotoxicity and inward flux of araC in KG1 cells.* AraC is an important agent used in the treatment of AML, ALL and MDS. In KG1 cells, the cytotoxic effects of araC were examined by flow cytometry assays. The KG1 cells were sensitive to the cytotoxic effects of araC, yielding an $IC_{50}$ value of 6.8 nM. Previous studies have reported the $IC_{50}$ values for araC cytotoxicity in OCI/AML-2 (a continuous line of AML myeloblasts) and CCRF/CEM cells to be 5.2 nM and 0.98 nM respectively.[74]

In KG1 cells, araC cytotoxicity was pharmacologically manipulated by the use of the nucleoside transport inhibitor, NBMPR. NBMPR significantly protected the cells from the cytotoxic effects of araC through blockade of *es* sites, indicating that nucleoside transporter activity was a determinant of araC sensitivity in KG1 cells. Both dilazep and DPM also protected the cells from the cytotoxic effects of araC, which was reflected in the $IC_{50}$ values, which were approximately 3-fold (dilazep) and 10-fold (DPM) higher than the $IC_{50}$ values in the absence of the transport inhibitors. This protection from araC

cytotoxicity by transport inhibitor, NBMPR has previously been observed in CCRF/CEM cells.[74]

The present study also showed that the effects of NBMPR and dilazep in protection against araC cytotoxicity were concentration dependent (Figure 5). The $IC_{50}$ values for protection by NBMPR and dilazep were 19 (6.6–54) nM and 12 (2.3–60) nM, respectively. It was also observed that these nucleoside transport inhibitors, alone, did not have any effect on the cell viability. Similar results have been found in CEM cells and freshly isolated leukemic cells from patients.[74] Together, these results suggest that NBMPR, apparently through the blockade of *es* transporter sites, protected the cells from the cytotoxic effects of araC, thereby indicating that membrane transport was a determinant of araC cytotoxicity under the conditions tested.

It should be noted that dilazep significantly protected KG1 cells from the cytotoxic effects of araC in a concentration dependent manner (Figure 5). This could be attributed to the effect of graded concentrations of dilazep employed for protection against a single concentration of araC (50nM). On the other hand, dilazep at the concentration of 1 μM did not significantly protect KG1 cells against the cytotoxicity of graded concentrations of araC, as shown in Table 1. Therefore, the effects of dilazep might not be significant at the higher concentrations of araC used in the latter experiments, where other araC uptake processes such as passive diffusion might be playing a role in araC cytotoxicity.

Inward fluxes of 0.8 μM araC were measured in KG1 cells by oil stop centrifugation technique (Figure 6). The araC concentration used (0.8 μM) lies in the range of araC concentrations achieved in plasma, when conventional doses of araC are given.[64] As shown in Figure 6, the fluxes of [$^3$H]araC were linear at least to 300 seconds, allowing a

wide range of time points for initial rate measurements. The *es* mediated component of flux was identified by the effects of NBMPR, which inhibited the flux of [$^3$H]araC in KG1 cells to a significant extent. The inhibition of influx of araC by NBMPR has previously been demonstrated in leukemic myeloblasts[29] and transformed hamster (MCT) cells.[97] These results suggested that *es* transporter contributes to the uptake of araC to a significant extent in KG1 cells.

A kinetic characterization of araC fluxes in KG1 cells was also performed. The uptake of araC in KG1 cells was saturable yielding the kinetic constants $K_m$, 240 ± 53 μM and $V_{max}$, 57 ± 4.2 pmol [$^3$H]araC/$10^6$ cells/sec/μM respectively. Furthermore, in the presence of 1 μM NBMPR, the inward flux of [$^3$H]araC was abolished. This result was apparently attributable to the blockade of *es* transporter sites in KG1 cells by NBMPR. Considering the precision of the experiment (n = 6), *es* nucleoside transporter might be the only transporter that facilitated araC transport in KG1cells.

A previous study[97] described the uptake of araC in MCT cell line (cell line derived from a tumor in hamsters after inoculation of $10^6$ ham cells (20-methylcholanthrene transformed hamster embryo cells) and reported the kinetic constants: $K_m$ = 350 μM and $V_{max}$ 780 μM· $min^{-1}$, which are comparable to the present values found in KG1 cells. This study also demonstrated that NBMPR inhibition of araC influx in KG1 cells was concentration dependent. With increasing concentrations of the transport inhibitor, NBMPR (0-1 μM) in the permeant solution, a progressive inhibition of the inward flux of [$^3$H]araC was evident, with $IC_{50}$ value of 12 (3.1-50) nM (Figure 8). With the increasing concentrations of NBMPR, an inhibition of araC flux in ovarian carcinoma cell line (CI 80-13S) has been demonstrated with $IC_{50}$ value of 4 nM.[98]

*Inward fluxes and Cytotoxicity of dFdC in KG1 cells.* The nucleoside analog, dFdC, has been useful in the treatment of certain solid tumors. However, resistance to dFdC has been apparent in patients with acute leukemia. This work has investigated the possibility that resistance to dFdC is associated with a deficiency in nucleoside transport. The cytotoxicity of dFdC and the modulation of dFdC cytotoxicity by NBMPR were studied in KG1 Cells (Figure 9). NBMPR significantly protected the cells from the cytotoxic effects of dFdC with $IC_{50}$ value 41 (21-78) nM, which was statistically significantly different from the $IC_{50}$ in the absence of NBMPR (4.3 (2.8-6.7) nM). The marked cytotoxic effects exerted by dFdC in KG1 cells and the protection from dFdC cytotoxicity by NBMPR suggests that *es* nucleoside transporters mediated dFdC cytotoxicity in KG1 cells. These results are consistent with those of Mackey *et al.*, who studied the cytotoxic effects of dFdC in CEM (transporter competent) and CEM-AraC-8C (nucleoside transporter incompetent cloned derivative, selected by resistance to 8 μM araC) cell lines. Nucleoside transporter defective, CEM-AraC-8C cells exhibited high levels of resistance to the drug and it was also inferred that dFdC uptake was mediated by *es*, *ei*, *cit* and *cib* nucleoside transporters in human leukemia cell lines.[18]

In the present study, inward fluxes of [$^3$H]dFdC were measured in KG1 cells, yielding initial rates of [$^3$H]dFdC uptake in the absence and presence of NBMPR. In the presence of 1 μM NBMPR, influx of 0.8 μM [$^3$H]dFdC was virtually abolished, indicating that dFdC (0.8 μM) uptake in KG1 cells was entirely attributable to (Figure 10) *es* nucleoside transporter activity. These results and the protection by NBMPR against dFdC cytotoxicity are consistent with the view that the *es* nucleoside transporter is a determinant of sensitivity to dFdC in KG1 cells. In KG1 cells, the inward flux of

[$^3$H]dFdC (5.4 ± 0.70 fmol [$^3$H]dFdC/$10^6$ cells/sec) was approximately 2.6 fold less as compared to the flux of [$^3$H]araC (14 ± 0.71 fmol [$^3$H]dFdC/$10^6$ cells/sec). However, NBMPR (1 μM) inhibited the flux of [$^3$H]dFdC and [$^3$H]araC up to 13-fold and 9-fold respectively.

It has been observed that the nucleoside transport activities varied distinctly in their apparent efficiencies of dFdC permeation among the cell lines with single nucleoside transporters: *es* ≅ *cit* > *ei* > *cib* >>> *cif*.[18] In another study the cytotoxic effects of dFdC were studied in three human pancreatic cell lines (PANC-1, HS-766T, and PK-8) and one human bladder cell line (MGH-U1).[82] That study hypothesized that these cell lines with more *es* transporters should display increased cytotoxicity to dFdC. However, it was observed that *es* transporters levels alone did not provide a good measure of predicting dFdC cytotoxicity. These authors suggested that other factors such as cellular efflux and intracellular activation might be important in the determination of dFdC cytotoxicity.

*Cytotoxicity and inward fluxes of araC in leukemic blasts from patients.* To maximize the clinical relevance of this project, the cytotoxicity and inward fluxes of araC and dFdC were studied in freshly isolated leukemic blasts from patients. It was hypothesized that the *es* nucleoside transporter-mediated membrane flux is a determinant of cytotoxicity of the nucleoside analogs in leukemic blasts. A wide interpatient variation in sensitivity to araC was seen in AML blast samples, and $IC_{50}$ values for araC (Table 2) were 15-676 fold higher than in KG1 cells. In 19 of 20 AML blast samples studied, the $IC_{50}$ values for araC cytotoxicity ranged between 0.05 – 4.6 μM (Mean ± SEM, 1.6 ± 0.37, n = 19). Gati *et al.* observed considerable interpatient variation in sensitivity to araC in blasts isolated from patients with AML, and a statistically significant correlation between cellular *es*

nucleoside transporter content and sensitivity to araC was found.[16] Another study reported considerable interpatient variation in sensitivity to araC, teniposide, vincristine and 6-thioguanine in blasts from patients with pediatric ALL.[99] This interpatient variation in sensitivity to antileukemic drugs might contribute to the clinical variations in the responses observed among patients.

In araC cytotoxicity experiments, blockade of *es* sites was carried out by simultaneously exposing the cells to NBMPR at a concentration of 1 μM (Figure 12). This tactic offered protection against araC cytotoxicity in 19 of 20 AML blast samples (Table 2). The observed reduction in araC sensitivity in the presence of the *es* nucleoside transport inhibitor indicates that a deficiency in nucleoside transport might contribute to araC resistance.

Previous studies have shown that nucleoside transport inhibitors such as NBMPR, dilazep and DPM protected the freshly isolated AML blasts from the cytotoxic effects of araC.[16] In another study performed by Gati *et al.* [74] it was observed that 1 μM NBMPR protected CEM cells and freshly isolated leukemic blasts against araC cytotoxicity. The study demonstrated that the viability of the cells when treated with the araC and NBMPR combination was 85% of controls in CEM cells and 34-110% of controls in fresh leukemic blasts.

Inward fluxes of [$^3$H]araC were examined in fresh leukemic blasts from 13 AML patient samples. The results indicated large differences between the fluxes of araC among the patient samples. Also, the fluxes were 1.7-140 fold less in the leukemic blasts from patients as compared to the flux rates in KG1 cells, confirming a previous report that initial rates of araC transport were much slower in leukemic blasts from patient

samples than in human leukemic cell lines.[29] The wide scatter in araC inward fluxes was observed for both total araC uptake as well as uptake attributable to the NBMPR-sensitive component. A wide interpatient variability has been observed previously in leukemic blasts from patient samples, especially the AML samples, which showed a 10-fold range of inward fluxes.[29] Up to 80-fold variation of araC influx among patient samples has also been reported.[100]

Inhibition of inward fluxes by NBMPR in leukemic blasts from AML patients (Table 5) confirms the presence of *es* nucleoside transporter activity. Previous studies by Wiley *et al.* have shown that membrane transport of araC in leukemic blasts is directly proportional to the abundance of [$^3$H]NBMPR binding sites, suggesting the role of *es* transport in the uptake of araC in those cells.[29] It has been demonstrated that at concentrations below 1 μM the rate of araC accumulation is determined by the transport rate.[17] The mean ± SD of the *es*-mediated flux in the blasts from the clinical samples was 1.3 ± 1.9, n=16. The possibility of uptake of araC by other NT processes could not be explored due to the limited number of cells isolated from each sample. Therefore, new methods for characterizing the nucleoside uptake in the cells need to be designed, which require fewer cells per assay.

Inward fluxes of araC measured in samples from patients with MDS or ALL yielded results that were similar to those in AML samples. Thus, a statistically significant inhibition of the flux in the presence of NBMPR was seen in some, but not all samples. As in the case of AML samples, araC fluxes in MDS and ALL cells were substantially lower than in KG1 cells (Table 6 and Table 7).

Previous studies have established a correlation between *es* nucleoside transporter abundance and cell sensitivity to araC in samples from patients with acute leukemia. A study performed by Gati *et al.* has shown that there existed a correlation between *es* nucleoside transporter content and sensitivity to araC in AML blasts from patients.[16] A statistically significant correlation between *es* transporter abundance and drug sensitivity was shown for araC but not for another nucleoside analog, 2-CdA in ALL blasts from pediatric patients.[92] It has also been shown that the formation of the cytotoxic metabolite of araC, araCTP was correlated with the number of nucleoside transporter sites in leukemic blasts from patients.[15] Altogether, these previous studies have established an association between the abundance of *es* nucleoside transporters and the cytotoxicity of araC in fresh leukemic blasts.

The present study compared the nucleoside transporter mediated inward flux of araC and the cytotoxicity of araC in fresh leukemic blasts from patients. It was hypothesized that there exists a correlation between the *es* nucleoside transporter-mediated membrane flux of araC and araC cytotoxicity. Such a correlation would strengthen the idea that a deficiency in *es* nucleoside transporter-mediated membrane flux of araC may be a predictor of araC resistance in leukemia. Flow cytometry was used to determine the araC sensitivity of the cells, which were stained with Cy-Chrome™ labeled anti-CD45, to identify and enumerate the leukemic blasts. It should be noted that [$^3$H]araC fluxes were measured in the entire low density "mononuclear" cell population isolated by Ficoll-Paque centrifugation whereas the cytotoxicity data were obtained from the leukemic blast population, gated and enumerated by flow cytometry.

A statistically significant positive correlation was established between the *es* nucleoside transporter mediated membrane flux of 0.8 μM araC and the NBMPR-sensitive component of araC (1 μM) cytotoxicity. This result suggests that the *es* nucleoside transporter-mediated membrane flux of araC is a determinant of araC cytotoxicity in fresh leukemic blasts from patients. Therefore, the function of the *es* transporter correlated with the cytotoxicity of araC in blasts from patients, suggesting that a deficiency in membrane transport might play a significant role in resistance to nucleoside analogs such as araC.

*Cytotoxicity and inward fluxes of dFdC in leukemic blasts from patient samples.* The cytotoxicity of dFdC was also evaluated in fresh leukemic blasts from patients. Most of the AML samples studied were resistant to dFdC, as $IC_{50}$ values were greater than 10 μM, the highest concentration of dFdC tested, both in the absence and presence of NBMPR. In 4 of the 8 AML samples, the leukemic cells were relatively sensitive to the cytotoxic effects of dFdC and NBMPR significantly protected the cells from the dFdC cytotoxicity in 3 of 8 samples. The $IC_{50}$ values for dFdC cytotoxicity were in the range of 0.015 – 4.4 μM and those for araC were in the range of 0.05 – 4.6 μM in fresh AML blasts. However, the clinically achievable concentrations of araC after continous intravenous infusion are 0.06-0.8 μM.[64]

Similar results were obtained in a small number of ALL blast samples. However, an interesting observation was the potentiation by NBMPR of the cytotoxic effects of dFdC in one sample (Table 9). The $IC_{50}$ value in the presence of NBMPR was reduced to 1.9 μM from > 10 μM, in the absence of the transport inhibitor. This potentiation of dFdC cytotoxicity might occur through the blockade of *es* transporter-mediated efflux of

dFdC by NBMPR, without affecting NBMPR-insensitive concentrative nucleoside transporters, which might be expressed in these cells. A previous study reported the cytotoxic effects of dFdC on leukemic blasts from chronic myeloid leukemia (CML) patients.[101] It was observed that dFdC had prominent inhibitory effects on the blasts from CML patients, with an apparent $IC_{50}$ of 4 nM.[101] In the present study, due to a limited number of samples available, a detailed characterization of dFdC cytotoxicity, and the protection by transport inhibitors such as NBMPR, could not be performed.

Inward fluxes of [$^3$H]dFdC fluxes were examined in fresh leukemic blasts from 8 leukemia patients. The fluxes [$^3$H]dFdC ranged from 0.19-1.3 fmol [$^3$H]dFdC/$10^6$ cells/sec and were 4-28 fold lower in the leukemic blasts as compared to KG1 cells. NBMPR has no significant effect on the [$^3$H]dFdC flux rates in 2 of the 4 AML samples studied.

Measurement of both dFdC cytotoxicity and inward fluxes was performed in only 3 samples because of limitations in the number of cells available for these experiments. As a result, no correlation between flux and cytotoxicity was attempted. Nevertheless, results of dFdC cytotoxicity and inward flux experiments suggest that nucleoside transport is a less important factor in dFdC cytotoxicity than in araC cytotoxicity in leukemia cells. However, further studies are warranted. Also, this study only examined the role of the *es* transporter in dFdC sensitivity in leukemic blasts from patients. The role of other transporters in dFdC cytotoxicity needs to be studied in detail. This might provide some insight into the mechanisms of dFdC resistance in leukemia.

*Selection and characterization of es nucleoside transporter deficient cell population.* For the past few years dFdC is employed in the treatment of solid malignancies such as

advanced pancreatic cancer.[78] The activity of dFdC has also been tested in hematological malignancies. Studies have shown that dFdC demonstrates good cytotoxic activity in human leukemic cell lines but its clinical activity in leukemia is still questionable.[81] Nucleoside transporters play a vital role in the transport of nucleoside analogs across the cell membrane, which is a prerequisite for their cytotoxicity. Therefore, nucleoside transporter deficiency might be a mechanism of resistance to nucleoside analogs such as dFdC. The objective of this part of thesis was to select a dFdC resistant, *es* transporter-deficient population of leukemic T-lymphoblasts and characterize the selected cell population. A T-lymphoblast cell line (CEM/CCL1) was employed as these cells had been selected earlier in our laboratory using FACS to isolate the cells dimly stained with SAENTA-fluorescein. The CEM/CCL1 cells were partly *es* deficient (approximately 4 fold reduction in *es* transporter abundance as compared to CCRF/CEM cells, Table 13) and were shown to be somewhat resistant to araC.

The cells were exposed to stepwise increasing concentration of dFdC. Upon exposure to the drug, the sensitive cells could not survive the treatment whereas the resistant ones survived. With each increase in dFdC concentration, a rise in the MGT of the cells was observed (Figure 18). However, the concentration of dFdC was not further increased till the MGT had again descended to the normal values. The selection procedure was terminated at 500 nM and the cells were maintained in medium containing 500 nM dFdC, and harvested and assayed from time to time (CEM/CCL1/G cells).

The *es* nucleoside transporter sites in CEM/CCL1/G cells were determined by utilizing a flow cytometric technique. A fluorescent probe, 5-(SAENTA-x8)-fluorescein, which binds to the NBMPR binding site on the *es* transporter was employed.[39] Previous

studies have employed SAENTA-fluorescein to determine the *es* transporter content in cell lines and in clinical samples.[16] As shown in Figure 19, the specific binding of SAENTA-fluorescein in CEM/CCL1/G cells was found to be approximately 7-fold less than in the CEM/CCL1 cells, from which they were selected. In order to determine whether the deficiency of *es* transporters was subject to the presence of dFdC in the culture medium, the cells were washed free of the drug (CEM/CCL1/Gw cells) and their *es* transporter abundance was measured from time to time over a period of 60 days. The specific binding of SAENTA-fluorescein in CEM/CCL1/G and CEM/CCL1/Gw cells was not statistically significantly different during this period. Thus, it was possible to isolate a cell population of T-lymphoblasts, which exhibited a stable, *es* transporter deficiency to dFdC in the absence of the drug used for selection.

The cell cycle phase distribution of the CEM/CCL1/G cells was studied to examine any changes in the growth properties of the selected cells. No statistical significant difference was observed in any cell cycle phases between CEM/CCL1, CEM/CCL1/G and CEM/CCL1/Gw cells (Figure 21). Thus, the cell cycle phase distribution of the selected cells did not undergo any significant changes as a result of the selection procedure.

Cross-resistance to other nucleosides including dFdC, araC, 2-CdA and dThd was examined in both CEM/CCL1 and CEM/CCL1/G cells. All the nucleosides, dFdC, araC, 2-CdA and dThd, require the *es* transporter for their transport into the cells. 2-CdA was chosen for the assay as it requires *es* nucleoside transporter for entry into the cells and like araC and dFdC, also requires dCK for activation. On the other hand dThd does not require dCK for activation but does require the *es* nucleoside transporter for its entry into

the cells. Cytotoxicity assays employing these drugs might provide a useful insight in understanding possible additional mechanisms of resistance to dFdC in the selected cell line. The cytotoxicity assay results showed that the dFdC selected cell line, CEM/CCL1/G, was > 12-fold and > 24-fold resistant to araC and dFdC respectively with the $IC_{50}$ values > 100 nM, whereas the CEM/CCL1/G cells were > 4-fold and ~3-fold resistant to dThd and 2-CdA respectively (Table 13). This 4-fold resistance to dThd could be attributed to a deficiency in *es* nucleoside transporter in the selected cells. Thus, these results indicate that *es* nucleoside transporter deficiency contributed largely to resistance to dFdC, araC, 2-CdA and dThd in CEM/CCL1/G cells.

In the cytotoxicity experiments, the dFdC and araC concentration-effect curves appeared to be biphasic in nature for the CEM/CCL1/G cells. A sensitive component in CEM/CCL1/G cells was evident, which was approximately 23% and 16% for dFdC and araC respectively (Figure 22). This sensitivity in CEM/CCL1/G cells could be attributed to the residual *es* nucleoside transporter content that was detected in the CEM/CCL1/G cells (Figure 19).

The enzyme, dCK plays an essential role in the conversion of nucleosides such as araC, dFdC and 2-CdA to their cytotoxic metabolites. Apart from a deficiency in *es* transporter abundance, dCdK deficiency could attribute to resistance to nucleoside drugs in CEM/CCL1/G cells. Deoxycytidine kinase activity has been shown to be decreased or absent in variants of human leukemic cell line K562, resistant to nucleoside analogs.[102] Some *in vitro* models have demonstrated cross-resistance between 2-CdA, araC and dFdC with reduced levels of dCK.[102] In order to elucidate the possible mechanisms of drug resistance in CEM/CCL1/G cells, dCK activity was measured in these cells and in

CCRF/CEM cells and CEM/CCL1 cells (Figure 24). The activity of dCK was found to be 54% in CEM/CCL1/G cells as compared to the controls (CEM/CCL1 cells), suggesting that a deficiency in dCK may have partly contributed to resistance to dFdC, araC and 2-CdA in the CEM/CCL1/G cells. It is likely that the major determinant of the drug resistance in the selected cells was the *es* transporter deficiency. However this was reduced to < 5% of the value in CCRF/CEM cells (Table 13), whereas the dCK activity was reduced to about 55% of CCRF/CEM value. It should also be noted that the 12-fold resistance to araC and 24-fold resistance to dFdC in CEM/CCL1/G cells could only be attributable to 7-fold reduction in the *es* transporter abundance in CEM/CCL1/G cells (as compared to CEM/CCL1 cells) as compared to the ~ 2-fold reduction in the dCK levels in the CEM/CCL1/G cells.

In summary, results from the present study indicate that *es* nucleoside transporter is the only transporter that mediates fluxes of araC and dFdC in KG1 cells. The protection against cytotoxicity by nucleoside transport inhibitors was apparently attributable to the blockade of inward fluxes of araC and dFdC in KG1 cells. Previous studies have established a correlation between *es* transporter abundance and cytotoxicity of nucleoside analogs such as araC.[16] This project has demonstrated a correlation between the *es* transporter mediated membrane flux of araC and cytotoxicity in leukemic blasts from patients. However, no correlation could be established between *es* mediated membrane flux and dFdC cytotoxicity in leukemic blasts from patients. It was possible to select an *es* nucleoside transporter-deficient, dFdC-resistant population of cells from a human leukemia cell line. Drug resistance in these cells could be attributed largely to the

deficiency of *es* nucleoside transporters, although dCK deficiency might be partly playing a role. These results together indicate the significance of nucleoside transporter mediated membrane flux of nucleoside analogs in cancer chemotherapy, and that the membrane flux of nucleoside analogs may play a significant role in resistance to these agents in leukemia cells.

*Future Directions.*

The characterization of other nucleoside transporters such as concentrative nucleoside transporters would further improve the understanding of the role of transport processes in leukemic blasts from patients. It may be possible to manipulate nucleoside fluxes pharmacologically by the use of nucleoside transport inhibitors in leukemic cells that express multiple nucleoside transport processes. For example, the expression of a concentrative nucleoside transporter such as *cib* would allow the cellular influx of nucleoside drugs, other than araC, in the presence of transport inhibitors, which would inhibit the equilibrative nucleoside transporter-mediated efflux.

In the present study, dFdC cytotoxicity could not be correlated with the *es* mediated membrane flux. The transport studies of dFdC in the *es* nucleoside transporter deficient cell line might confirm the role of *es* transporter in the uptake of dFdC by showing whether the dFdC resistant, CEM/CCL1/G cells demonstrate altered membrane fluxes of dFdC. The intracellular metabolism of dFdC in leukemic blasts needs to be studied in detail in order to elucidate the mechanisms of dFdC resistance in leukemia.

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